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Regulation of Behavioral Arousal in *C. elegans*

A dissertation presented

by

Seungwon Choi

to

the Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Genetics

Harvard University

Cambridge, Massachusetts

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Regulation of Behavioral Arousal in *C. elegans*

ABSTRACT

Animals undergo periods of behavioral quiescence and arousal in response to environmental, circadian, or developmental cues. During larval molts, *C. elegans* undergoes a period of profound behavioral quiescence termed lethargus. Locomotion quiescence during lethargus was abolished in mutants lacking a neuropeptide receptor (NPR-1), and was reduced in mutants lacking NPR-1 ligands (FLP-18 and -21). Wild type strains are polymorphic for the *npr-1* gene, and their lethargus behavior varies correspondingly. Locomotion quiescence and arousal were mediated by decreased and increased secretion of an arousal neuropeptide (PDF-1) from central neurons. PDF receptors (PDFR-1) expressed in peripheral mechanosensory neurons enhanced touch-evoked calcium transients. Thus, a central circuit stimulates arousal from lethargus by enhancing the sensitivity of peripheral mechanosensory neurons in the body. These results define a circuit mechanism controlling a developmentally programmed form of quiescence.

Sensory experience is critical for structural and functional plasticity of neural circuits and proper behavioral responses to environmental stimuli. Cholinergic transmission drives body muscle activity and modulates locomotion in *C. elegans*. Cholinergic transmission at neuromuscular junctions was enhanced in mutants lacking NPR-1, which was mediated by heightened central sensory circuit activity. Both

glutamate and neuropeptide were required for the sensory-evoked potentiation of cholinergic transmission. Thus, a central sensory circuit activity increases excitatory transmission in the peripheral body muscles through a concerted action of glutamate and neuropeptide. These results define a circuit mechanism underlying sensory-evoked alteration in synaptic activity and locomotion.

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Chapter 1

Introduction

Animals coordinately adjust their behaviors in response to changes in their environment and metabolic state. Co-regulated behaviors (often termed behavioral states) can persist for minutes to hours. Increased activity (or arousal) is associated with fear, stress, hunger, and exposure to sexual partners (Pfaff et al., 2008a). Conversely, decreased activity (or quiescence) is associated with sleep and satiety (Cirelli, 2009).

Circadian and homeostatic control of behavioral arousal

Behavioral arousal is characterized by increase in motor activity, responsiveness to sensory stimuli, and emotional reactivity (e.g. motivation) (Pfaff, 2006). These aspects of behavioral arousal exhibit rhythmic patterns associated with circadian rhythm, a biological rhythm that oscillates with a period of approximately 24 hours (Allada and Chung, 2010). For example, nocturnal animals (e.g. rodents) exhibit aroused behaviors such as increased locomotion, olfactory responsiveness, and motivation to find food during nighttime, while diurnal animals (e.g. flies and humans) do during daytime. Disruption of circadian rhythm by lesion of suprachiasmatic nucleus (SCN) in hypothalamus, a master clock in mammals, or by mutations inactivating circadian genes alters timing and quality of behavioral arousal (Cirelli, 2009).

The mechanism underlying changes in behavioral arousal is best studied in sleep/wakefulness cycle (Silver and Lesauter, 2008). The dominant model for sleep regulation is the two-process model, where sleep and wakefulness is regulated by two factors, circadian rhythm and homeostatic pressure (Borbely, 1982). Homeostatic pressure increases proportionately with the time spent awake and decreases with the time spent asleep. Therefore, if wakefulness lasts for an extended time, homeostatic pressure

increases and triggers sleep rebound to compensate for sleep deprivation. On the other hand, circadian rhythm determines the phase - onset and duration - of sleep, thereby promoting consolidation of sleep in one major phase. Thus, disruption of circadian rhythm causes phase shift or fragmentation of sleep (Cirelli, 2009).

Neuropeptide regulation of behavioral arousal

Behavioral arousal of animals is modulated by multiple neurotransmitters. Cholinergic and noradrenergic activities positively regulate cortical activation and alertness (Jones, 2008), and dopamine signaling is essential for motivated behaviors (Palmiter, 2008). In particular, neuropeptides play a key role in modulating brain and behavioral states (Pfaff et al., 2008b).

Among the neuropeptides that modulate behavioral states, hypocretin/orexin peptides have the most profound effect on behavioral arousal, controlling sleep and wakefulness in vertebrates (Sutcliffe and de Lecea, 2002). The importance of hypocretin/orexin in regulating sleep/wakefulness first came from the genetic linkage study with a canine model of narcolepsy, where a mutation in hypocretin/orexin receptor 2 was identified to cause narcolepsy in dogs (Lin et al., 1999). Similarly, hypocretin/orexin knockout mice exhibit narcoleptic behavior (Chemelli et al., 1999), while hypocretin/orexin overexpression triggers insomnia-like behavior in zebrafish (Prober et al., 2006). Human narcolepsy is also associated with hypocretin/orexin deficiency (Nishino et al., 2000; Peyron et al., 2000). Together, these studies suggest that hypocretin/orexin acts as a wake-promoting ‘arousal’ peptide in vertebrates.

Another peptide that affects behavioral states in mammals is corticotropin-releasing factor (CRF). CRF signaling activates hypothalamic-pituitary-adrenal (HPA) axis and mediate endocrinal and behavioral arousal responses to stress (Bale and Vale, 2004). Overexpression of CRF in mice induces anxiety-like behavior (Stenzel-Poore et al., 1994), whereas mice deficient for CRF receptor 1 exhibit decreased anxiety and impaired stress response (Smith et al., 1998; Timpl et al., 1998). Moreover, central administration of CRF increases sleep latency and decreases sleep duration in rats (Ehlers et al., 1997). These studies suggest that CRF largely favors aroused state in mammals.

In contrast to restricted expression of hypocretin/orexin in hypothalamus, Neuropeptide Y (NPY) is broadly expressed throughout the brain, and regulates diverse physiological function such as food intake, anxiolysis, heart rate, and pain transmission. In addition, NPY modulates behavioral arousal as well (Dyzma et al., 2010). For example, administration of NPY into the brain decreases locomotor activity in rodents (Heilig and Murison, 1987; Jolicoeur et al., 1991). Similarly, NPY administration reverses the wake-promoting effect of CRH (Ehlers et al., 1997) in rats, and increases sleep duration and decreases sleep latency and wake time in humans (Antonijevic et al., 2000; Held et al., 2006). Moreover, NPY reduces spike frequency and hyperpolarize the membrane potential of hypocretin/orexin neurons in mouse hypothalamus, which serve as a wake-promoting center in the mammalian brain (Fu et al., 2004). These studies suggest that NPY inhibits behavioral arousal in mammals.

NPR-1 (Neuropeptide Receptor), a NPY receptor homolog in *C. elegans*

The *npr-1* gene encodes a predicted G protein-coupled receptor (GPCR) homologous to mammalian NPY receptors (de Bono and Bargmann, 1998). *C. elegans* NPR-1 shares ~30% sequence identity and ~50% sequence similarity with mammalian NPY receptors. Despite the homology between NPR-1 and NPY receptors, NPY-like sequences do not exist in the *C. elegans* genome. Likewise, human NPY does not activate G-protein signaling in NPR-1 expressing heterologous cells (Kubiak et al., 2003). Instead, FMRFamide (Phe-Met-Arg-Phe-NH₂)-related peptides (FaRPs), FLP-18 and FLP-21, bind and activate NPR-1 in transfected heterologous cells (Kubiak et al., 2003; Rogers et al., 2003). It has been reported that FaRPs can also serve as ligands for NPY-like receptors in flies and mammals (Feng et al., 2003; Hinuma et al., 2000).

Polymorphism in the *npr-1* gene

NPR-1 was first identified whose natural variants caused distinct foraging behaviors in *C. elegans* (de Bono and Bargmann, 1998). *C. elegans* natural isolates harboring valine at 215th amino acid position of NPR-1 (NPR-1 215V) exhibit solitary foraging behavior and disperse across a bacterial lawn. By contrast, natural isolates containing phenylalanine at the same position (NPR-1 215F) exhibit social foraging behavior and aggregate together on bacterial lawn. Thus, the *npr-1* gene is polymorphic among wild type populations, with 215F allele being more frequently found (McGrath et al., 2009; Weber et al., 2010). 215th amino acid of NPR-1 resides at the border of 5th transmembrane domain and 3rd intracellular loop that is critical for G-protein coupling. Consistently, the two NPR-1 wild type alleles encode receptors that differ in their affinity for NPR-1 ligands (FLP-18 and

FLP-21), with 215V exhibiting higher affinity (~4 fold) and lower EC50's (~40 fold) than 215F receptors (Kubiak et al., 2003; Rogers et al., 2003). Consequently, inactivating NPR-1 ligand FLP-21 in worms expressing low affinity (and high EC50's) NPR-1(215F) receptors had much greater effect on foraging behavior than inactivating FLP-21 in worms expressing high affinity (and low EC50's) NPR-1(215V) receptors (Rogers et al., 2003). Recent studies also suggested a possibility that 215V allele might be a laboratory-derived polymorphism (McGrath et al., 2011; Weber et al., 2010).

Role of NPR-1 in *C. elegans* behaviors

npr-1 is expressed predominantly in the nervous system, in which its expression is largely concentrated in neurons in the sensory circuit (Coates and de Bono, 2002; de Bono and Bargmann, 1998). Expressed in sensory circuit, NPR-1 regulates diverse sensory behaviors, as evidenced by altered behavioral responses to environmental cues such as oxygen, carbon dioxide, pheromone, and pathogen in *npr-1* mutant animals (Bretscher et al., 2008; Cheung et al., 2004; Cheung et al., 2005; Coates and de Bono, 2002; Gray et al., 2004; Hallem and Sternberg, 2008; Macosko et al., 2009; Reddy et al., 2009; Srinivasan et al., 2012; Styer et al., 2008). For example, repulsion from hyperoxia and attraction to certain types of pheromone is exaggerated in *npr-1* mutants, which contributes to social foraging behavior (Cheung et al., 2005; Gray et al., 2004; Macosko et al., 2009; Srinivasan et al., 2012). Similarly, pathogen susceptibility is also increased in *npr-1* mutants, as evidenced by decreased survival rate when exposed to *Pseudomonas aeruginosa* PA14 (Reddy et al., 2009; Styer et al., 2008). Blocking sensory transduction with mutations inactivating sensory transduction genes (CNG, TRPV channels, and

guanylate cyclase) or ablation of corresponding sensory neurons abolish all these altered sensory behaviors in *npr-1* mutants. These results suggest that NPR-1 normally inhibits activity of sensory neurons, such that inactivating NPR-1 causes increase in sensory activities. Consistent with this idea, NPR-1 is coupled to G_i/G_o proteins and hyperpolarizes membrane potential through G protein-activated inwardly rectifying K⁺ channels (GIRKs) (Rogers et al., 2003).

Hub-and-spoke model for social foraging behavior of *npr-1* mutants

NPR-1 regulates diverse sensory modalities and related behaviors. How can a single gene activity alter such a diverse behavioral outputs? *C. elegans* nervous system is wired with highly stereotyped synaptic connections, which consists of 5000 chemical synapses, 2000 neuromuscular junctions (NMJs) and 600 gap junctions (White et al., 1986). In the head sensory circuit, the RMG interneurons form gap junctions with several classes of sensory neurons (Fig 1.1). NPR-1 is expressed in a subset of these gap junction circuit (the RMG circuit) neurons including RMG (Fig 1.1), thereby controlling the sensory transduction occurring in the RMG circuit. Consistent with this idea, transgenes expressing NPR-1 in RMG neurons rescue the *npr-1* foraging defect (aggregation and increased speed), while ablating RMG neurons abolishes the *npr-1* defect (Macosko et al., 2009). Similarly, mutations inactivating NPR-1 increase pheromone-evoked calcium responses in ASK sensory neurons, even though ASK neurons do not express NPR-1.

Interestingly, the sensory neurons anatomically coupled to RMG largely mediate responses to environmental repellents. For example, ASH neurons are polymodal nociceptive neurons that mediate responses to nose touch, high osmolarity, and aversive

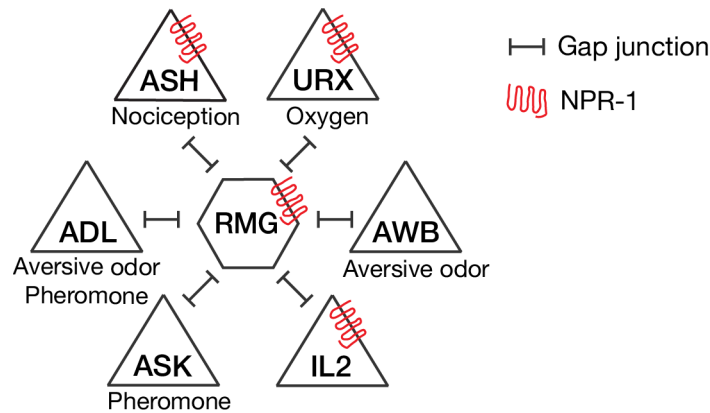


Figure 1.1. A schematic illustrating the RMG circuit.

Sensory neurons (triangles) mediating the indicated aversive responses form direct gap junctions with the RMG interneuron (hexagon). Cells expressing NPR-1 are indicated (Coates and de Bono, 2002; Macosko et al., 2009). This diagram is modified from that shown previously (Macosko et al., 2009).

chemicals (Hilliard et al., 2004; Kaplan and Horvitz, 1993; Sambongi et al., 1999). URX neurons mediate avoidance from hyperoxic environment (Cheung et al., 2005; Gray et al., 2004). Repulsion from aversive odors is mediated by ADL and AWB neurons (Troemel et al., 1995; Troemel et al., 1997). The choice between attraction and repulsion responses to different types of pheromones is mediated by ASK, ASI, and ADL neurons (Jang et al., 2012; Kim et al., 2009; Macosko et al., 2009; McGrath et al., 2011). The *npr-1* foraging defect is suppressed by inactivating ion channels such as TAX-4/CNG, OSM-9 and OCR-2/TRPV channels that mediate sensory transduction in these sensory neurons (Coates and de Bono, 2002; de Bono et al., 2002; Macosko et al., 2009). These results suggest that altered sensory transduction in the RMG circuit causes the foraging defect in *npr-1* mutants. Collectively, NPR-1 can regulate ‘spoke’ sensory neurons and related behavioral outputs by altering the activity of ‘hub’ RMG neurons.

Conserved role of NPY in inhibiting neural activity

Most NPY receptors are also coupled to G_i/G_o proteins and hyperpolarize membrane potential through activation of K^+ currents mediated by GIRK channels and inhibition of Ca^{2+} currents mediated by voltage-gated calcium channels in rodent brain (Fu et al., 2004; Sun and Miller, 1999; Sun et al., 2001). For example, NPY inhibits hypocretin/orexin neurons in hypothalamus, thereby tonically attenuating hypocretin-regulated arousal (Fu et al., 2004). In thalamus, NPY reduces activity of reticular thalamic neurons, thereby suppressing thalamic network oscillations (Sun et al., 2003), which is relevant to sleep and arousal (Steriade et al., 1993). Thalamus processes sensory information and relays the sensory signals to the associated cortical area, whose function is analogous to that of the RMG neurons in *C. elegans* (Fig 1.1). Thus, both NPY receptors and NPR-1 modulate sensory inputs by controlling the activity of sensory-gatekeeping neurons, which are thalamic neurons in mammals and the RMG neurons in *C. elegans*.

Dissertation overview

Chapter 2

In chapter 2, I describe a circuit mechanism controlling molting-associated behavioral quiescence and arousal in *C. elegans*. During larval molts, *C. elegans* undergoes a period of profound behavioral quiescence termed lethargus. A recent study provided several pieces of evidence that lethargus is a sleep-like state in *C. elegans* (Raizen et al., 2008), which is characterized by behavioral quiescence (feeding and movement), reduced responsiveness to external stimuli, and homeostatic rebound quiescence after perturbation.

We provide evidence that locomotion quiescence during lethargus is specifically abolished in mutants lacking a neuropeptide receptor (NPR-1), whereas feeding quiescence is normal. We then propose a neuropeptide-mediated circuit mechanism underlying ‘insomnia’ phenotype exhibited by *npr-1* mutants. A series of experimental results based on behavioral analyses, imaging, and calcium recordings suggest that sensory-coupled secretion of neuropeptide PDF-1 from the RMG circuit dictates behavioral state through its action on PDF receptors (PDFR-1) expressed in peripheral mechanosensory neurons.

I then discuss conserved role of functional NPY homologues as a ‘quiescence peptide’, and PDF homologues as an ‘arousal peptide’ in worms, flies, and rodents. I also discuss neuropeptide-mediated coupling of central sensory circuit to peripheral motor circuit. Altered PDF-1 secretion from central neurons engenders rhythmic locomotor activity associated with molting through activation of PDF receptors functioning in touch neurons and body muscles.

Chapter 3

Sensory experience modulates the function of neural circuits and behavioral outputs. In chapter 3, I describe a circuit mechanism controlling sensory-evoked enhancement of excitatory synaptic transmission at *C. elegans* NMJs. While I focus on behavioral outcome of altered sensory activity in *C. elegans* in chapter 2, I focus more on sensory input-driven change in synaptic activity in a defined neural circuit in chapter 3. I begin with an introduction of a couple of examples showing sensory experience-dependent plasticity in neural circuits studied in mammals. Then I briefly describe the structure of

nervous system in *C. elegans*, and pharmacological and electrophysiological tools we utilized to measure body muscle activity.

We provide evidence that cholinergic transmission is enhanced in mutants lacking NPR-1. We then propose a circuit mechanism underlying sensory-evoked potentiation of cholinergic transmission. A series of experimental results based on both pharmacological and electrophysiological assays suggest that central sensory circuit activity increases excitatory transmission in the peripheral body muscles through a concerted action of glutamate and neuropeptide.

I then discuss future experiments for identification of downstream target cells of glutamate signaling and potential neuropeptides that may mediate sensory-evoked increase in body muscle activity. I also discuss physiological role of sensory-evoked enhancement of cholinergic transmission at NMJs.

Chapter 4

In chapter 4, I comment on the implications of our findings, with an emphasis on sensory-evoked and neuropeptide-mediated regulation of neural circuit and behavior. I also comment on preliminary results and future experiments that must still be done to answer the following questions; (1) what sensory modalities promote arousal from lethargus? (2) Does lethargus regulate NPR-1 and the ligands? (3) What is the downstream effector of PDFR-1? (4) Does body muscle activity change during other forms of behavioral quiescence? (5) What is the pathogen effect on arousal?

Chapter 2

Analysis of NPR-1 Reveals a Circuit Mechanism for Behavioral Quiescence in *C. elegans*

This chapter contains work published as Seungwon Choi, Marios Chatzigeorgiou, Kelsey P. Taylor, William R. Schafer, and Joshua M. Kaplan (2013), which was in press in *Neuron* at the time of writing.

Marios Chatzigeorgiou performed all of the touch-evoked calcium recordings. Seungwon Choi and Kelsey Taylor performed RNAi screen together. Seungwon Choi performed all of the other experiments. Seungwon Choi and Joshua Kaplan assembled the manuscript, with input from William Schafer and Marios Chatzigeorgiou.

INTRODUCTION

Many aspects of behavior and metabolism exhibit rhythmic patterns with a periodicity of approximately 24 hours, which are generically referred to as circadian rhythms (Allada and Chung, 2010). Daily behavioral and metabolic rhythms are accompanied by a corresponding set of circadian changes in gene expression. Circadian rhythms are dictated by a cell autonomous clock that consists of a transcriptional feedback network that exhibits intrinsically oscillating activity. The period of this circadian clock is entrained by daily changes in light and temperature; although, daily rhythms persist even in constant conditions. Thus, circadian clocks provide a mechanism that allows animals to couple their behavior to anticipated changes in their environment.

Rhythmic changes in behavior and metabolism are also often coupled to developmental clocks. In the nematode *C. elegans*, molting exhibits a rhythmic pattern with a periodicity of 8-10 hours. This molting cycle is dictated by cell intrinsic developmental clock genes (termed heterochronic genes) (Moss, 2007). The periodicity of the molting cycle is dictated by rhythmic changes in the expression of a heterochronic gene (*lin-42*), which is homologous to the fly circadian gene PERIOD (Jeon et al., 1999; Monsalve et al., 2011). Thus, circadian and heterochronic clocks are mediated by similar biochemical mechanisms.

Although a great deal is known about the biochemical and genetic mechanisms controlling circadian and heterochronic timing, relatively little is known about how these clocks are coupled to changes in behavior, i.e. to their outputs. To address this question, we analyzed the rhythmic behaviors associated with the *C. elegans* molting cycle.

During each larval molt, *C. elegans* undergoes a prolonged period of profound behavioral quiescence, whereby locomotion and feeding behaviors are inactive for ~2 hours. This molt associated quiescence is termed lethargus behavior, and has been described for many wild type nematode species (Cassada and Russell, 1975). Lethargus has properties of a sleep-like state such as reduced sensory responsiveness and homeostatic rebound of quiescence following perturbation (Raizen et al., 2008). Several genes and molecular pathways involved in lethargus behavior have been identified (Monsalve et al., 2011; Raizen et al., 2008; Singh et al., 2011; Van Buskirk and Sternberg, 2007); however, a circuit mechanism controlling lethargus associated quiescence has not been defined.

Here we identify a central sensory circuit that dictates entry into and exit from locomotion quiescence during lethargus. Quiescence is associated with decreased activity in this central circuit, while arousal is associated with increased circuit activity. This central circuit regulates motility through the action of a neuropeptide (Pigment Dispersing Factor-1, PDF-1), which enhances the sensitivity of peripheral mechanosensory receptors in the body. These results provide a circuit mechanism controlling arousal and quiescence of locomotion in *C. elegans*.

RESULTS

Locomotion quiescence during lethargus is blocked in *npr-1* mutants

Mutants lacking the neuropeptide receptor NPR-1 have heightened responsiveness to oxygen and pheromones, which results in altered foraging behavior and accelerated locomotion (Cheung et al., 2005; Gray et al., 2004; Macosko et al., 2009). Thus, NPR-1 is proposed to set the threshold for arousal of specific behaviors. Prompted by these results, we tested the idea that NPR-1 also regulates arousal from behavioral quiescence during lethargus. To analyze animals during the L4 to adult (L4/A) lethargus, we isolated a synchronous population of L4 animals and analyzed their behaviors during the subsequent molt. As in wild type animals, the pharyngeal pumping of *npr-1* mutants was completely arrested during the L4/A lethargus (Fig. 2.1A). The duration of pharyngeal

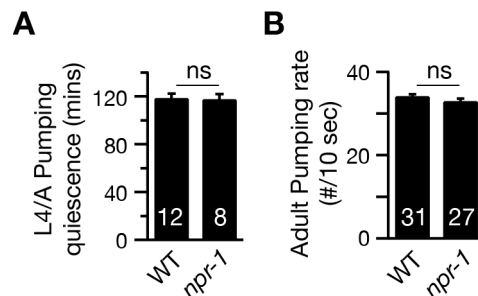


Figure 2.1. *npr-1* mutants have normal feeding behavior.

Pharyngeal pumping was analyzed in the indicated genotypes. (A) The duration of feeding quiescence during the L4/A lethargus (defined by the absence of pharyngeal pumping) was unaltered in *npr-1* mutants. (B) Adult pharyngeal pumping rate was also unaltered in *npr-1* mutants. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. (ns, not significant).

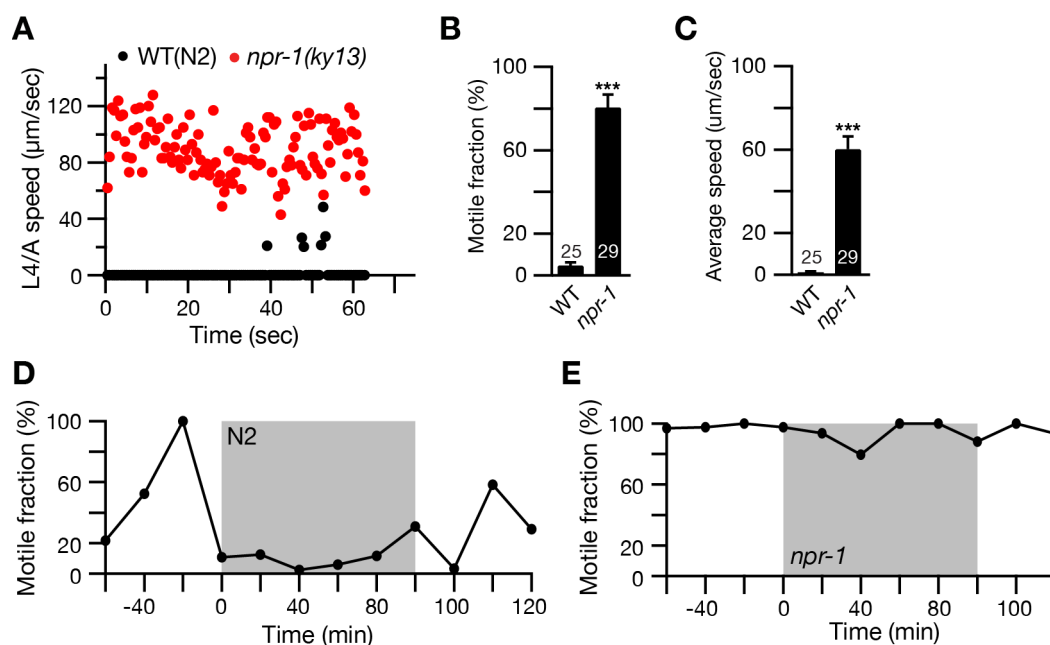


Figure 2.2. NPR-1 regulates locomotion quiescence during lethargus.

(A-C) Locomotion behavior of single worms during the L4/A lethargus was recorded for 60-75 seconds and velocity was measured (2 Hz sampling). Instantaneous locomotion velocity (A), average motile fraction (B), and average locomotion velocity (C) are plotted. *npr-1* null mutants had higher locomotion during the L4/A lethargus than WT (N2). (D-E) Representative traces of motile fraction during L4/A lethargus are shown for wild type (D) and *npr-1* mutants (E). Gray boxes indicate lethargus, as determined by absence of pharyngeal pumping. Locomotion of *npr-1* mutants was continuously active during lethargus. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

pumping quiescence was unaltered in *npr-1* mutants, indicating that the duration of lethargus had not been altered (Fig. 2.1A). Pharyngeal pumping rate was also unaltered in *npr-1* adults (Fig. 2.1B). To assess changes in locomotion during the L4/A lethargus, we analyzed the fraction of time animals undergo active motility (motile fraction) and

locomotion velocity. Unlike wild type animals, *npr-1* mutants exhibited fast and nearly continuous locomotion during the L4/A lethargus (Fig. 2.2A-C). The effects of *npr-1* on locomotion persisted throughout the entire L4/A lethargus (as defined by pumping quiescence) (Fig. 2.2D-E). Inactivation of *npr-1* had a significantly larger effect on locomotion during the L4/A lethargus (motile fraction, 17-fold increase; velocity, 50-fold increase) than in adults (motile fraction, 1.2-fold increase; velocity, 2-fold increase) (Fig. 2.3). These results suggest that NPR-1 is required for locomotion quiescence during lethargus, but not for feeding quiescence.

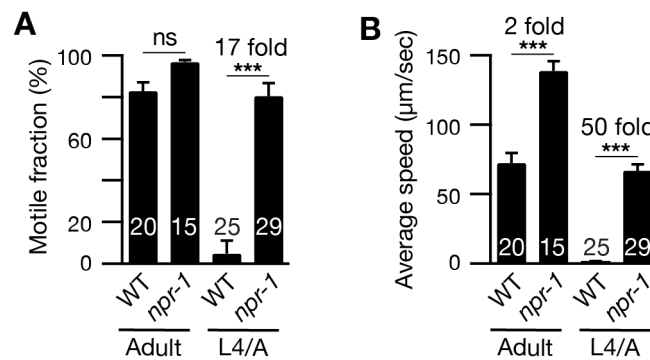


Figure 2.3. Inactivation of *npr-1* has a larger effect on locomotion during the L4/A lethargus than in adults.

Locomotion of adults and animals during L4/A lethargus were compared. Motile fraction (A) and average speed (B) are shown. During lethargus, *npr-1* mutants had a 17-fold increase in motile fraction and a 50-fold increase in average speed, whereas significantly smaller changes were observed in adults (motile fraction 1.2-fold increase, $p = 0.24$; speed 2-fold increase, $p < 0.001$). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$; ns, not significant).

Wild type strains are polymorphic for lethargus locomotion behavior

The *npr-1* gene is polymorphic among wild type populations, with two frequent alleles observed (215V and 215F) (McGrath et al., 2009; Weber et al., 2010). These wild type alleles encode receptors that differ in their affinity for NPR-1 ligands (FLP-18 and FLP-21), with 215V exhibiting higher affinity (and lower EC₅₀'s) than 215F receptors (Kubiak et al., 2003; Rogers et al., 2003). To determine if wild type strains are also polymorphic for lethargus behavior, we analyzed locomotion during the L4/A lethargus (Fig. 2.4). All 215V containing strains exhibited similar levels of quiescence and were

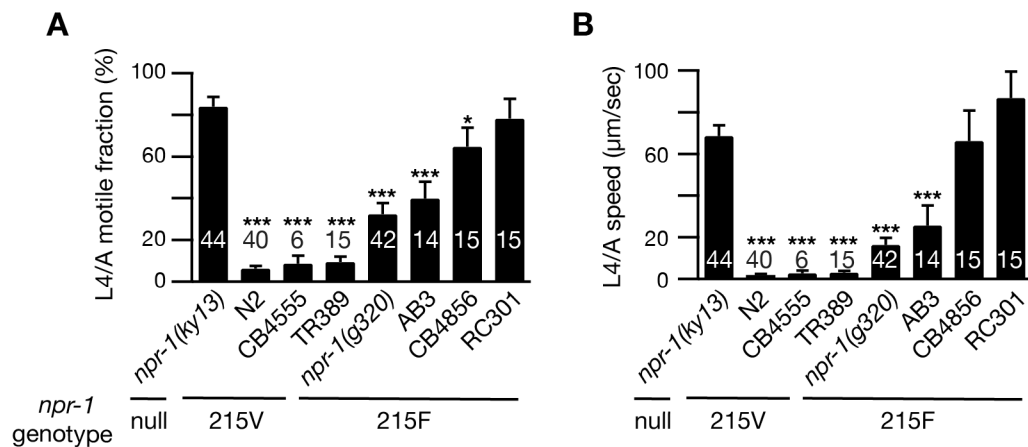


Figure 2.4. Wild type strains are polymorphic for lethargus locomotion behavior.

Locomotion behavior of single worms during the L4/A lethargus was recorded for 30-75 seconds and velocity was measured (2 Hz sampling). Average motile fraction (A), and average locomotion velocity (B) are plotted. Wild type strains were polymorphic for L4/A locomotion, with 215V strains being more quiescent than 215F strains. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated ((*, $p < 0.05$; ***, $p < 0.001$).

significantly more quiescent than 215F strains. The quiescence observed in 215F strains was more variable, with one strain (RC301) exhibiting L4/A locomotion similar to *npr-1* null mutants while others (AB3 and CB4856) exhibited intermediate levels of quiescence. Thus, the extent of behavioral quiescence during lethargus is polymorphic among wild type strains. A strain carrying a 215F allele (*g320*) in the Bristol genetic background had significantly stronger quiescence than was observed in unrelated 215F wild type strains (e.g. CB4856 and RC301). These results suggest that variation in genes other than *npr-1* also contributed to differences in the lethargus behaviors of wild type strains.

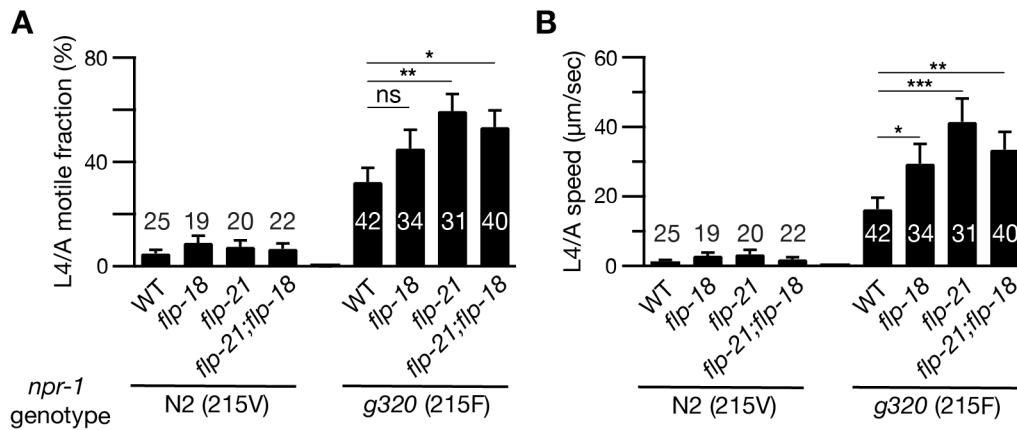


Figure 2.5. The NPR-1 ligands FLP-21 and FLP-18 regulate lethargus behavior.

Locomotion behavior of single worms during the L4/A lethargus was recorded for 30-75 seconds and velocity was measured (2 Hz sampling). Average motile fraction (A), and average locomotion velocity (B) are plotted. Mutations inactivating NPR-1 ligands, FLP-18 and FLP-21, decreased L4/A locomotion quiescence in animals expressing NPR-1(215F) receptors, but not in those expressing NPR-1(215V) receptors, i.e. *npr-1(g320)* mutants and N2 respectively. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly from *npr-1(g320)* L4/A are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant).

The NPR-1 ligands FLP-21 and FLP-18 regulate lethargus behavior

Two NPR-1 ligands have been identified, the neuropeptides FLP-18 and FLP-21 (Kubiak et al., 2003; Rogers et al., 2003). Both neuropeptides bind and activate NPR-1 receptors expressed in transfected cells; however, NPR-1 exhibits significantly stronger affinity for FLP-21. We found that mutations inactivating FLP-18 and FLP-21, and double mutants inactivating both ligands, had no effect on the L4/A locomotion behavior of worms expressing high affinity NPR-1(215V) receptors (Fig. 2.5). By contrast, inactivating either FLP-18 or FLP-21 significantly decreased locomotion quiescence in a Bristol strain expressing low affinity NPR-1(215F) receptors, i.e. *npr-1(g320)* mutants (Fig. 2.5). These results suggest that FLP-18 and FLP-21 function as endogenous NPR-1 ligands to regulate lethargus behavior in strains expressing NPR-1(215F) receptors.

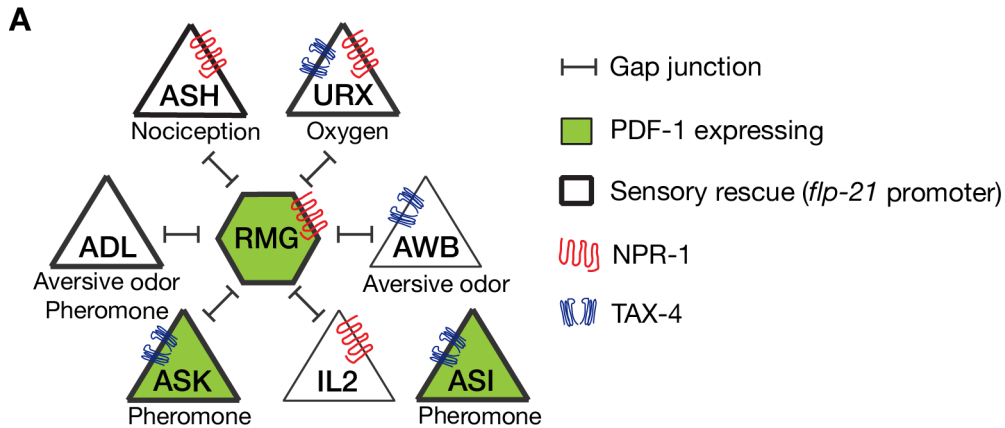


Figure 2.6. A diagram illustrating the RMG circuit.

Sensory neurons (triangles) mediating the indicated aversive responses form direct gap junctions with the RMG interneuron (hexagon). Cells expressing NPR-1, TAX-4/CNG channels, PDF-1, and the *flp-21* promoter (sensory rescue) are indicated (Barrios et al., 2012; Coates and de Bono, 2002; Janssen et al., 2009; Komatsu et al., 1996; Macosko et al., 2009; Rogers et al., 2003). ASI neurons are not directly connected to RMG but are also a potential source of PDF-1. This diagram is modified from that shown previously (Macosko et al., 2009).

The *npr-1* lethargus defect is mediated by increased sensory activity

NPR-1's effects on foraging are mediated by its expression in a sensory circuit in the head that is defined by gap junctions to the RMG interneuron (Fig. 2.6) (Macosko et al., 2009). Hereafter, we refer to this circuit as the RMG circuit. In addition to the RMG circuit, NPR-1 is also expressed in GABAergic motor neurons in the ventral nerve cord (Coates and de Bono, 2002). We did two experiments to determine where NPR-1 functions to regulate motility during lethargus. First, an *npr-1* transgene expressed in the RMG circuit (using the *flp-21* promoter) (Fig. 2.6) completely rescued the lethargus locomotion defect of *npr-1* mutants, whereas a transgene expressed in GABAergic motor neurons (using the *unc-30* promoter) had no rescuing activity (Fig. 2.7). Second, the

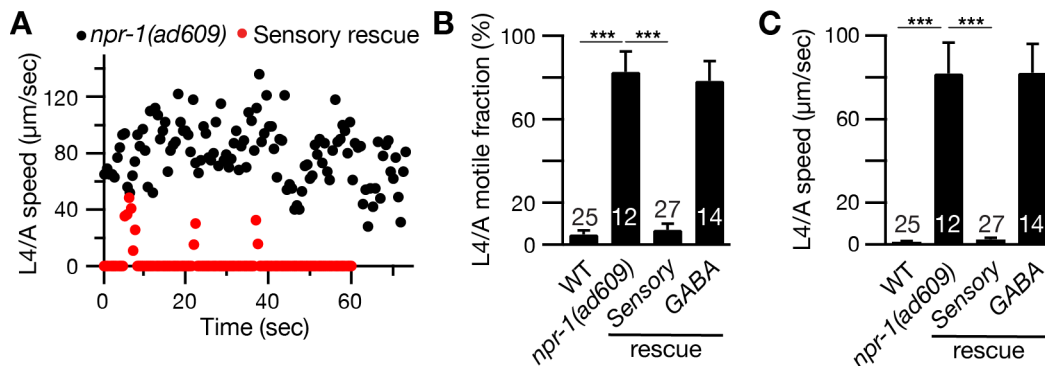


Figure 2.7. NPR-1 functions in the RMG circuit to regulate lethargus locomotion.

Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A), average motile fraction (B), and average locomotion velocity (C) are plotted. The *npr-1* L4/A locomotion quiescence defect was rescued by transgenes expressing NPR-1 in the RMG circuit (Sensory rescue, *flp-21* promoter) but not by those expressed in GABAergic neurons (GABA rescue, *unc-30* promoter), using the indicated promoters. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

lethargus locomotion defect of *npr-1* mutants was abolished by mutations inactivating ion channels required for sensory transduction, such as TAX-4/CNG and OSM-9/TRPV channels (Fig. 2.8). A transgene expressing TAX-4 in the RMG circuit re-instated the L4/A quiescence defect in *tax-4; npr-1* double mutants (Fig. 2.8). These results suggest

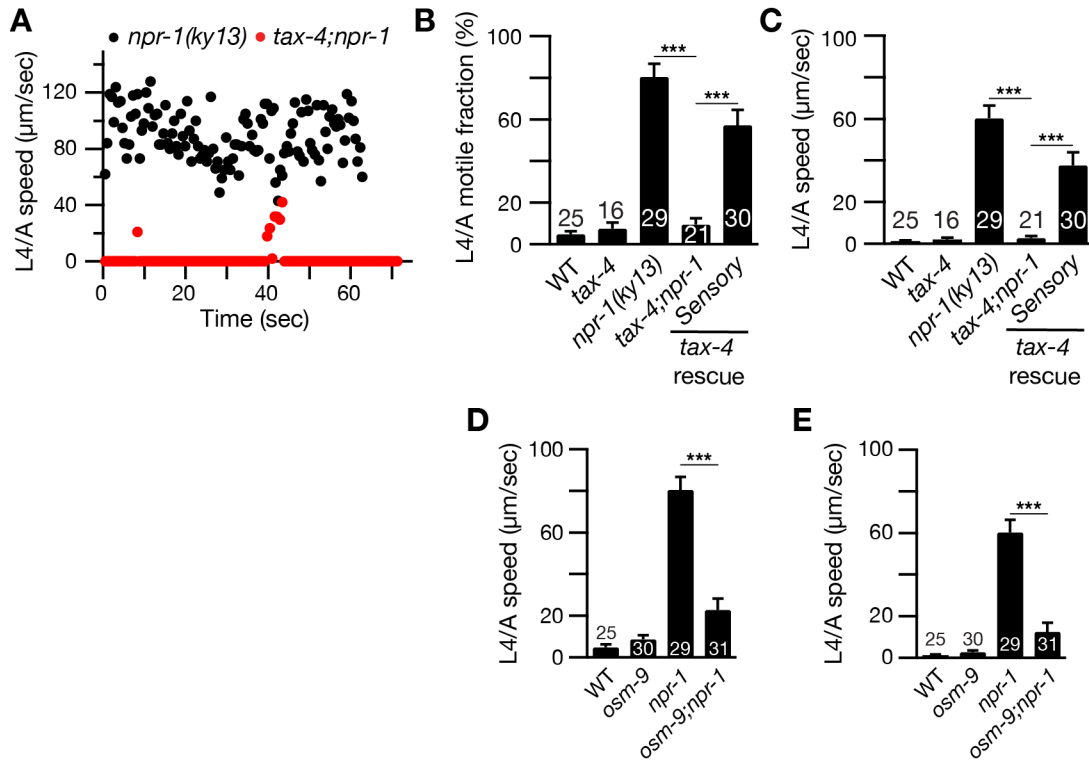


Figure 2.8. The *npr-1* lethargus defect is mediated by increased sensory activity.

Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A), average motile fraction (B,D), and average locomotion velocity (C,E) are plotted. (A-C) The *npr-1* L4/A locomotion quiescence defect was suppressed in double mutants lacking TAX-4/CNG channels and was reinstated by transgenes expressing TAX-4 in the RMG circuit (Sensory rescue, *flp-21* promoter). (D-E) The *npr-1* L4/A locomotion quiescence defect was also suppressed in double mutants lacking OSM-9/TRPV1 channels. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

that the *npr-1* defect in locomotion quiescence during lethargus was caused by heightened sensory activity in the RMG circuit.

PDF (Pigment Dispersing Factor) is required for the *npr-1* lethargus defect

Neuropeptides play a pivotal role in sleep and wakefulness in other systems. For example, hypocretin/orexin regulates sleep, arousal, feeding, and metabolism in vertebrates (Sutcliffe and de Lecea, 2002). Thus, we tested if neuropeptides are required for the *npr-1* lethargus defect. Consistent with this idea, the *npr-1* lethargus quiescence defect was eliminated by mutations inactivating *egl-3* PC2 and *pkc-1* PKC ϵ (Fig. 2.9), which are required for pro-neuropeptide processing and dense core vesicle (DCV) exocytosis, respectively (Husson et al., 2006; Kass et al., 2001; Sieburth et al., 2007).

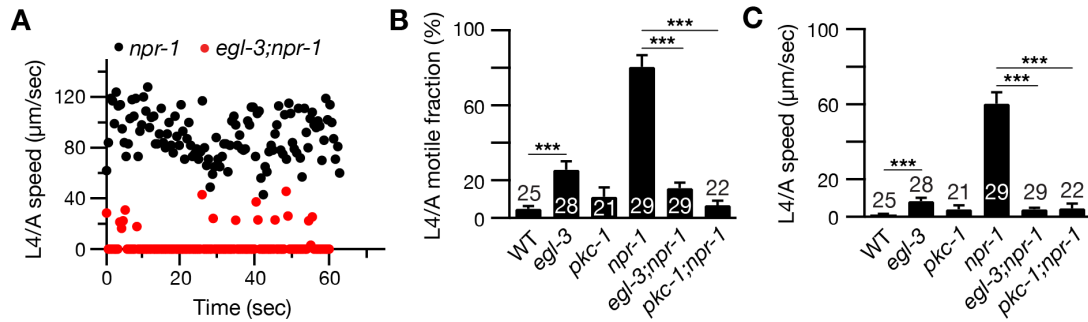


Figure 2.9. Neuropeptides mediate arousal from locomotion quiescence during lethargus.

Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A), average motile fraction (B), and average locomotion velocity (C) are plotted. The *npr-1* L4/A locomotion quiescence defect was suppressed by mutations that block neuropeptide processing (*egl-3* PC2 mutants) and dense core vesicle exocytosis (*pkc-1* PKC ϵ mutants). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

These results suggest that the *npr-1* lethargus defect was mediated by an endogenous neuropeptide.

In *Drosophila*, the neuropeptide Pigment Dispersing Factor (PDF) regulates circadian rhythms and promotes wakefulness (Parisky et al., 2008; Renn et al., 1999). Prompted by PDF's role in *Drosophila*, we tested the idea that PDF mediates the lethargus quiescence defect in *npr-1* mutants. *C. elegans* PDF peptides (PDF-1 and PDF-2) and their receptor (PDFR-1) were previously identified (Janssen et al., 2008; Janssen et al., 2009). PDF-1 is expressed in several classes of sensory neurons and interneurons, including ASK chemosensory neurons and RMG interneurons in the RMG circuit (Barrios et al., 2012; Janssen et al., 2009) (Fig. 2.6). The locomotion rate and motile fraction of *pdf-1;npr-1* and *pdfr-1;npr-1* double mutants during the L4/A lethargus were significantly lower than in *npr-1* single mutants (Fig. 2.10). Inactivating PDF-1 and

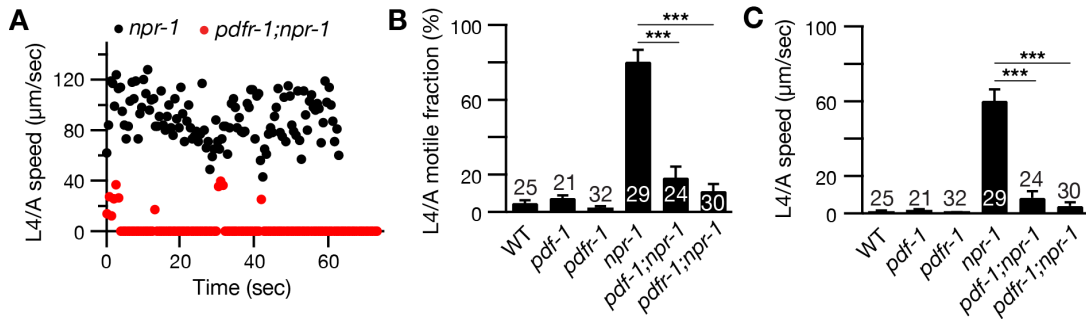


Figure 2.10. PDF-1 and PDFR-1 mediate arousal from locomotion quiescence during lethargus.

Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A), average motile fraction (B), and average locomotion velocity (C) are plotted. The *npr-1* L4/A locomotion quiescence defect was suppressed by mutations inactivating PDF-1 and PDFR-1. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

PDFR-1 had a much less dramatic effect on adult locomotion in *pdf-1;npr-1* and *pdf-1;npr-1* double mutants (Fig. 2.11). Thus, increased signaling by PDF-1 and PDFR-1 in *npr-1* mutants was required for the increased motility during lethargus. The *npr-1* foraging defect was unaltered in *pdf-1;npr-1* and *pdf-1;npr-1* double mutants (Fig. 2.11), indicating that PDF was not required for other *npr-1* phenotypes. Inactivating PDF-2 had

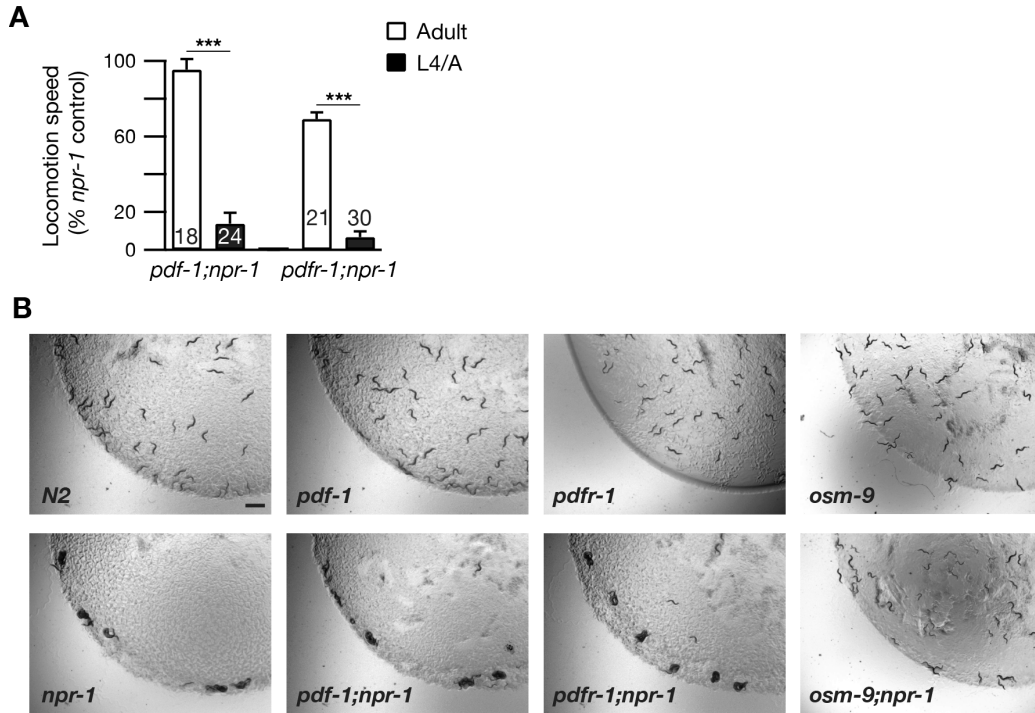


Figure 2.11. Inactivating PDF-1 and PDFR-1 have little effect on the *npr-1* defect of adult locomotion and foraging behavior.

(A) The change in average locomotion velocity (normalized to *npr-1* mutants) is summarized for the indicated genotypes. A *pdf-1* and *pdf-1* mutations had more dramatic effect on the *npr-1* locomotion during the L4/A lethargus than in adults. (B) Representative images of foraging behavior on bacterial lawns are shown for the indicated genotypes. Neither *pdf-1* nor *pdf-1* mutations prevented clumping of *npr-1* mutants, whereas clumping was abolished by an *osm-9* TRPV mutation as expected (de Bono et al., 2002). Scale bar indicates 1 mm. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$; ns, not significant).

little effect on the locomotion of *npr-1* mutants during lethargus (Fig. 2.12), indicating the PDF-1 is the major form of PDF involved in lethargus behavior. Collectively, these results suggest that PDF-1 functioned as an arousal peptide in *npr-1* mutants, preventing locomotion quiescence during lethargus. PDF-1's effects on arousal were specific because knockdown of 14 other neuropeptides expressed in the RMG circuit had no effect on the *npr-1* lethargus defect (Fig. 2.13).

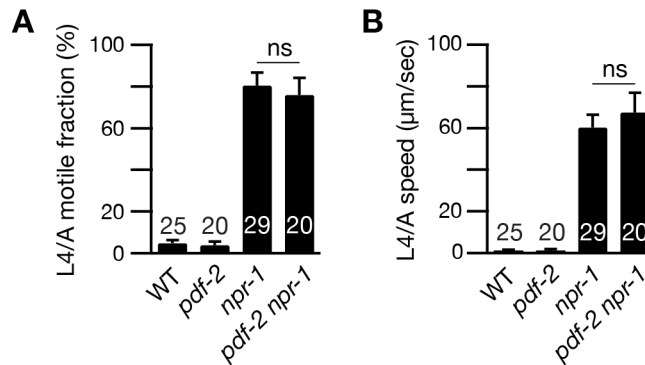


Figure 2.12. Inactivating PDF-2 does not suppress the *npr-1* lethargus locomotion defect.

Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Average motile fraction (A) and average locomotion velocity (B) are plotted. The *npr-1* locomotion quiescence defect during lethargus was not suppressed by inactivating PDF-2. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. (ns, not significant).

NPR-1 inhibits PDF-1 secretion during lethargus

If PDF-1 functions as an arousal peptide, PDF-1 expression or secretion should be inhibited during lethargus, when animals are quiescent. We did several experiments to test this idea. The abundance of *pdf-1* and *pdf-1* mRNAs (assayed by quantitative PCR) was unaltered during the L4/A lethargus, whereas expression of *mlt-10* (a gene required

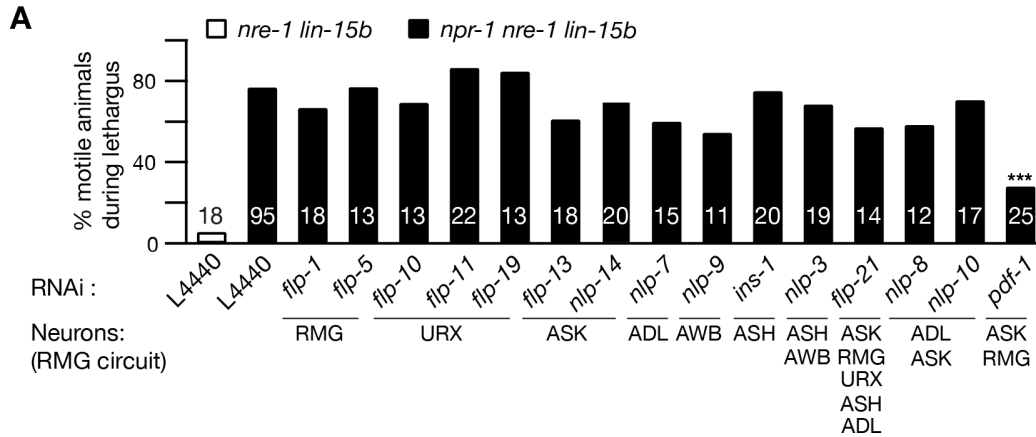


Figure 2.13. RNAi screen for the neuropeptide genes expressed in the RMG circuit whose knockdown suppresses the *npr-1* lethargus locomotion defect.

(A) The fraction of motile animals during the L4/A lethargus was plotted following treatment with the indicated RNAi clones. RNAi was carried out using RNAi hypersensitive strains (*nre-1 lin-15b*). Knockdown of *pdf-1* significantly suppressed the *npr-1* lethargus locomotion defect, whereas knockdown of 14 other neuropeptides expressed in the RMG circuit had no effect on the *npr-1* lethargus defect. L4440 indicates the empty vector control. The number of animals analyzed is indicated for each RNAi clone. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

for molting) was significantly increased, as expected (Fig. 2.14) (Frand et al., 2005). To assay PDF-1 secretion, we expressed YFP-tagged proPDF-1 with the *pdf-1* promoter (Fig. 2.15). During DCV maturation, the YFP linked to proPDF-1 is cleaved by proprotein convertases, and is subsequently secreted by DCV exocytosis. To assess the level of PDF-1 secretion, we analyzed PDF-1::YFP fluorescence in the endolysosomal compartment of coelomocytes, which are specialized scavenger cells that internalize proteins secreted into the body cavity (Fares and Greenwald, 2001; Sieburth et al., 2007). The PDF-1::YFP secretion reporter produced high levels of coelomocyte fluorescence in both L4 larvae and adults, whereas dramatically lower coelomocyte fluorescence was

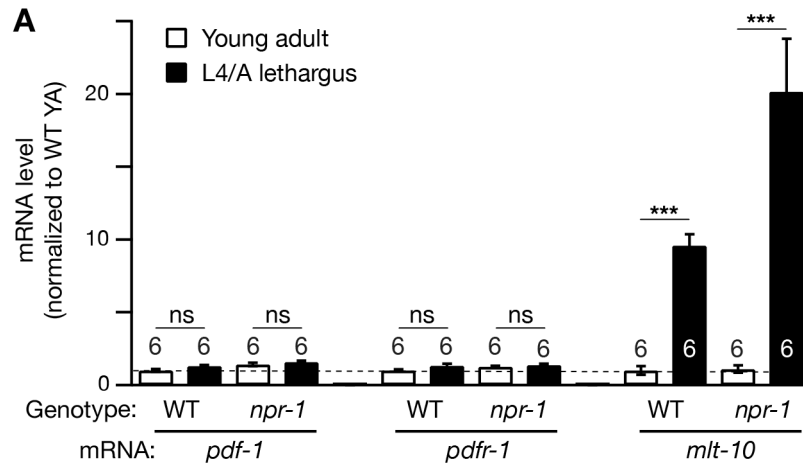


Figure 2.14. The abundance of *pdf-1* and *pdf-1* mRNAs is not altered during lethargus.

(A) The abundance of *pdf-1*, *pdf-1*, and *mlt-10* mRNAs in worm extracts was analyzed by quantitative PCR. For each gene, values reported were normalized to those observed in wild type young adults. The abundance of *pdf-1* and *pdf-1* mRNAs in lethargus and young adults were not significantly different, whereas *mlt-10* mRNA abundance was significantly increased during lethargus, as expected. 6 biological replicates were analyzed for each genotype and mRNA. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$; ns, not significant).

observed during the L4/A lethargus (Fig. 2.15). Coelomocyte fluorescence produced by a second secretion probe (mCherry-tagged RIG-3 expressed in cholinergic motor neurons) (Babu et al., 2011) was unaltered during lethargus (Fig. 2.16), indicating that secretion and coelomocyte function were not globally inhibited during lethargus.

If decreased PDF-1 secretion during lethargus is a cellular mechanism for inducing quiescence, we would expect that mutants retaining or lacking locomotion quiescence would exhibit reciprocal patterns of PDF-1 secretion during lethargus. We did several experiments to test this idea. In *npr-1* mutants, which lack quiescence, the decrease in

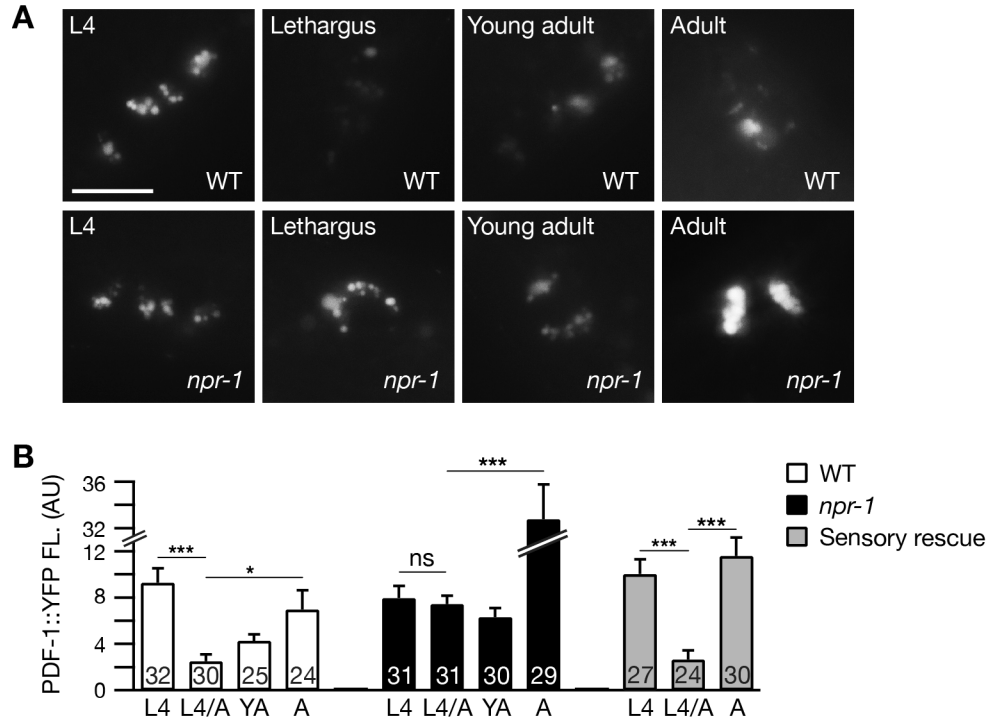


Figure 2.15. NPR-1 inhibits PDF-1 secretion during lethargus.

PDF-1 secretion (A,B) was analyzed in the indicated genotypes. (A,B) YFP-tagged PDF-1 was expressed with the *pdf-1* promoter. Representative images (A) and summary data (B) are shown for coelomocyte fluorescence in L4, L4/A, young adult (0-2 eggs in uterus), and gravid adults of the indicated genotypes. PDF-1::YFP coelomocyte fluorescence was dramatically reduced during the L4/A lethargus of wild type animals, but not in *npr-1* mutants. Decreased PDF-1::YFP coelomocyte fluorescence during lethargus was reinstated by transgenes expressing NPR-1 in the RMG circuit (Sensory rescue, *flp-21* promoter). The number of animals analyzed is indicated for each genotype. Scale bar indicates 10 μ m. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; ***, $p < 0.001$; ns, not significant).

PDF-1::YFP coelomocyte fluorescence during the L4/A lethargus was eliminated, and was restored by a transgene expressing NPR-1 in the RMG circuit (Fig. 2.15). Similarly, *tax-4*; *npr-1* double mutants exhibited locomotion quiescence and decreased PDF-1

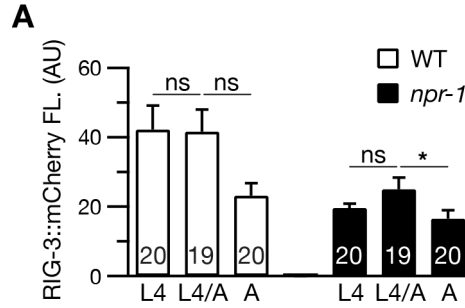


Figure 2.16. RIG-3 secretion is not reduced during lethargus.

(A) Coelomocyte fluorescence produced by RIG-3::mCherry (expressed in cholinergic neurons) was analyzed in L4, L4/A, and adult animals for the indicated genotypes. RIG-3::mCherry coelomocyte fluorescence was not altered during lethargus, and was reduced in L4 and L4/A *npr-1* mutant animals. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; ns, not significant).

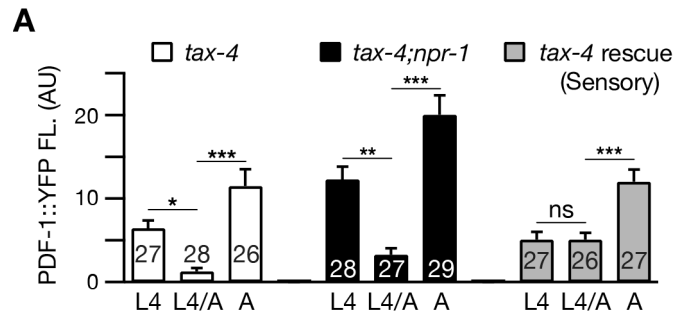


Figure 2.17. PDF-1 secretion is regulated by sensory activity in the RMG circuit.

(A) Coelomocyte fluorescence produced by PDF-1::YFP (expressed with the *pdf-1* promoter) was analyzed in L4, L4/A, and adult animals for the indicated genotypes. PDF-1::YFP coelomocyte fluorescence was decreased during the L4/A lethargus of *tax-4* single and *tax-4;npr-1* double mutants, which was reversed by transgenes expressing TAX-4 in the RMG circuit (Sensory rescue, *flp-21* promoter). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

secretion during lethargus, and both effects were reversed by a transgene expressing TAX-4 in the RMG circuit (Fig. 2.8A-C and 2.17). By contrast, RIG-3 coelomocyte fluorescence was decreased in *npr-1* mutants, in both L4 and L4/A animals (2.16). Consequently, the effects of NPR-1 and TAX-4 on PDF-1 coelomocyte fluorescence are unlikely to be caused by general changes in the stability of secreted proteins, nor by general changes in coelomocyte activity. Instead, these results suggest that heightened RMG circuit activity in *npr-1* mutants produced a corresponding increase in PDF-1 secretion from head sensory neurons, thereby increasing motility during lethargus. The preceding results suggest that decreased and increased PDF-1 secretion during lethargus are correlated with and required for locomotion quiescence and arousal. To

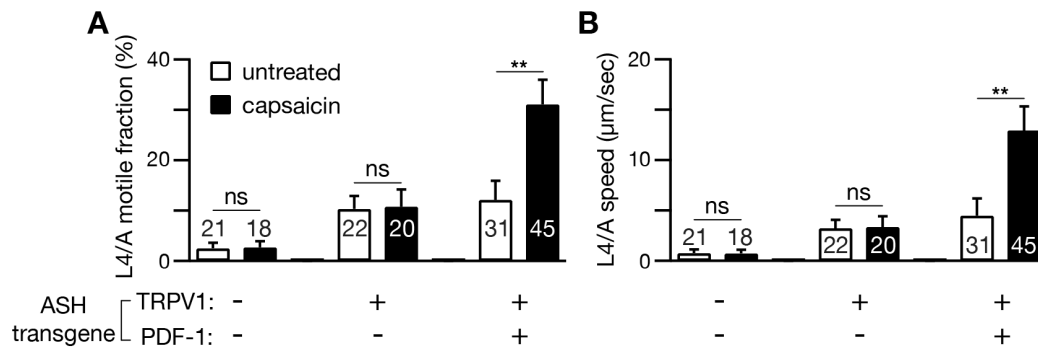


Figure 2.18. Forced secretion of PDF-1 during lethargus is sufficient to arouse locomotion behavior.

Forced depolarization of PDF-1 expressing neurons decreased L4/A locomotion quiescence. PDF-1 and rat TRPV1 were ectopically expressed in ASH neurons (using the *sra-6* promoter). Locomotion behavior of transgenic worms during the L4/A lethargus was analyzed with or without capsaicin treatment (6-7 hours). Average motile fraction (A), and average locomotion velocity (B) are plotted. Capsaicin treatment decreased L4/A quiescence in transgenic animals expressing both TRPV1 and PDF-1 in ASH neurons, but not in those expressing only TRPV1. The number of animals analyzed is indicated for each genotype. Values that differ significantly are indicated (**, $p < 0.01$; ns, not significant)

determine if increased PDF-1 secretion is sufficient to arouse locomotion, we constructed transgenic animals in which PDF-1 secretion can be pharmacologically induced (Fig. 2.18). A prior study showed that capsaicin treatment depolarizes ASH neurons expressing rat TRPV1 channels (Tobin et al., 2002). When TRPV1 and PDF-1 were co-expressed in ASH neurons, capsaicin treatment significantly decreased locomotion quiescence during lethargus (Fig. 2.18). This effect was not observed when only TRPV1 was expressed in ASH. These results suggest that forced secretion of PDF-1 during lethargus was sufficient to arouse locomotion behavior.

PDF-1 can function in ASK neurons to mediate arousal

Because RMG circuit activity controls PDF-1 secretion and locomotion arousal, a simple explanation for our data would be that PDF-1 is secreted by cells in the RMG circuit. Several results are consistent with this idea. The *pdf-1* promoter is expressed in RMG interneurons, and in ASK sensory neurons, which form direct gap junctions with RMG (Fig. 2.6) (Barrios et al., 2012; Janssen et al., 2008). Transgenes expressing PDF-1 in ASK neurons re-instated the locomotion quiescence defect in *pdf-1;npr-1* double mutants (Fig. 2.19A-B). Similarly, coelomocyte fluorescence produced by PDF-1::YFP expressed in ASK neurons was decreased during lethargus in wild type animals but not in *npr-1* mutants (Fig. 2.19C). Thus, PDF-1 expression in ASK neurons was sufficient to reconstitute NPR-1's effects on locomotion quiescence and PDF-1 secretion during lethargus. Because PDF-1 is secreted (and consequently acts in a cell non-autonomous manner), PDF-1 secretion from other cells may also regulate lethargus behavior.

Consistent with this idea, PDF-1 expression in ASI neurons also restored the L4/A quiescence defect in *pdf-1;npr-1* double mutants (Fig. 2.19A-B).

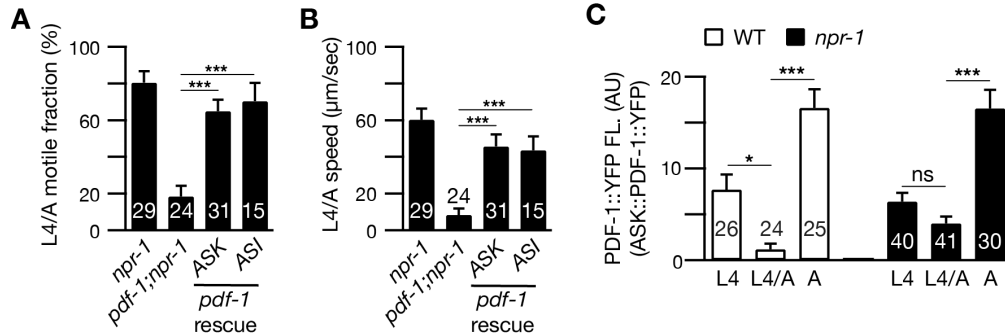


Figure 2.19. PDF-1 can function in ASK neurons to mediate arousal.

The effect of *pdf-1* rescue in ASK neurons on the *npr-1* lethargus locomotion defect (A-B) and PDF-1 secretion from ASK neurons (C) were analyzed in the indicated genotypes. (A-B) Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Average motile fraction (A), and average locomotion velocity (B) are plotted. The *npr-1* locomotion quiescence defect was reinstated in *pdf-1;npr-1* double mutants by transgenes expressing PDF-1 in ASK (*sra-9* promoter) and ASI (*str-3* promoter) sensory neurons using the indicated promoters. (C) YFP-tagged PDF-1 was expressed in ASK neurons using the *sra-9* promoter. Summary data (C) are shown for coelomocyte fluorescence in L4, L4/A, and gravid adults of the wild type and *npr-1* mutant animals. PDF-1::YFP coelomocyte fluorescence was reduced during the L4/A lethargus of wild type animals, but not in *npr-1* mutants. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; ***, $p < 0.001$; ns, not significant).

PDFR-1 acts in mechanosensory neurons to mediate arousal

How does enhanced PDF-1 secretion alter locomotion? The *pdf-1* promoter is expressed in mechanosensory neurons that sense vibration of the body wall (the touch neurons), in

body wall muscles, and in a few other classes of neurons (Janssen et al., 2008).

Transgenes expressing PDFR-1 in touch neurons or in body wall muscles both partially reinstated the lethargus locomotion quiescence defect in *npr-1*; *pdf-1* double mutants (Fig. 2.20A, C and D). These results suggest that PDFR-1 acts in both touch neurons and

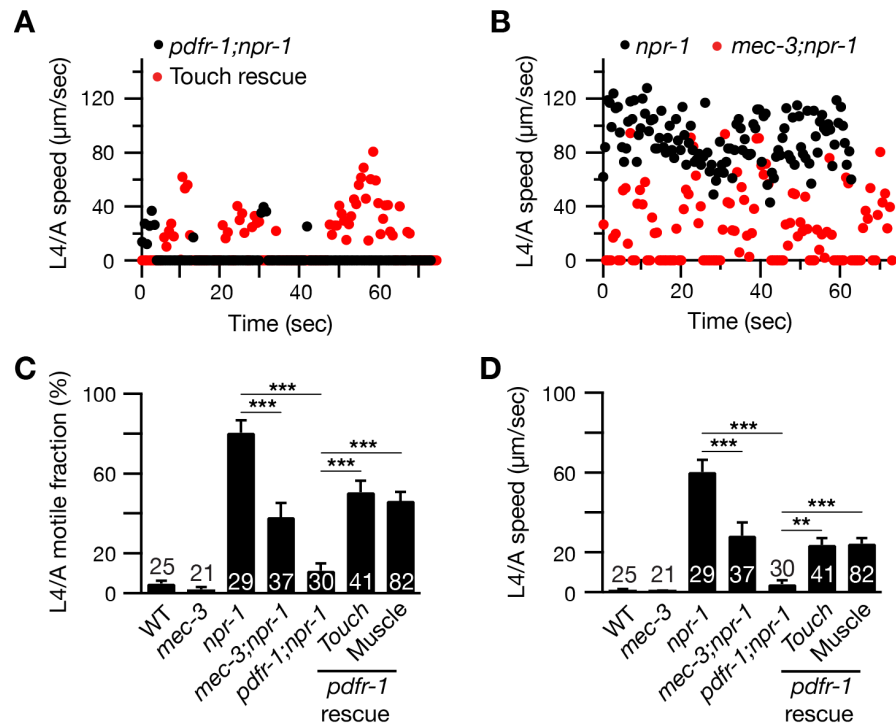


Figure 2.20. PDFR-1 receptors expressed in touch neurons and body muscles mediate locomotion arousal.

Locomotion behavior of single worms during the L4/A lethargus was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A,B), average motile fraction (C), and average locomotion velocity (D) are plotted. The *npr-1* locomotion quiescence defect was partially reinstated in *pdf-1*; *npr-1* double mutants by transgenes expressing PDFR-1 in touch neurons (*mec-3* promoter) and body muscles (*myo-3* promoter) using the indicated promoters (A,C,D). Mutations disrupting touch neuron differentiation (*mec-3* mutants) partially suppressed the *npr-1* locomotion quiescence defect (B-D). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (**, $p < 0.01$; ***, $p < 0.001$).

body wall muscles to promote arousal from locomotion quiescence during lethargus. The six touch neurons form gap junctions with the ventral cord command interneurons that control locomotion (Chalfie et al., 1985). Mutations that impair the mechanosensitivity of the touch neurons (termed Mec mutants) cause locomotion to become lethargic (Chalfie and Sulston, 1981). For these reasons, we focused our analysis on PDFR-1 function in touch neurons.

Is the *npr-1* lethargus defect mediated by increased activity of the touch neurons? We did several experiments to test this idea. First, we analyzed the lethargus behavior of *mec-3; npr-1* double mutants. The MEC-3 transcription factor is required for differentiation of touch neurons; consequently, touch responses are disrupted in *mec-3* mutants (Way and Chalfie, 1988). Mutations inactivating *mec-3* partially suppressed the lethargus locomotion defect of *npr-1* mutants (Fig. 2.20B-D). These results suggest that touch neuron function was required for NPR-1's effect on motility during lethargus. Partial suppression of the lethargus defect in *mec-3; npr-1* double mutants was expected because rescue experiments suggest that PDFR-1 function is required in both touch neurons and body muscles (Fig. 2.20A, C and D).

Second, we measured touch-evoked calcium transients in the anterior touch neuron (ALM) of adult animals using the genetically encoded calcium indicator cameleon (Fig. 2.21 and 2.22). Cameleon expression in touch neurons did not disrupt NPR-1 and PDFR-1 effects on L4/A locomotion quiescence (Fig. 2.23). Thus, calcium buffering by cameleon did not interfere with NPR-1 mediated regulation of touch cell function. PDF-1 secretion was increased in *npr-1* adults (Fig. 2.15); consequently, NPR-1's effects on touch sensitivity should be evident in adults. Consistent with this idea, the

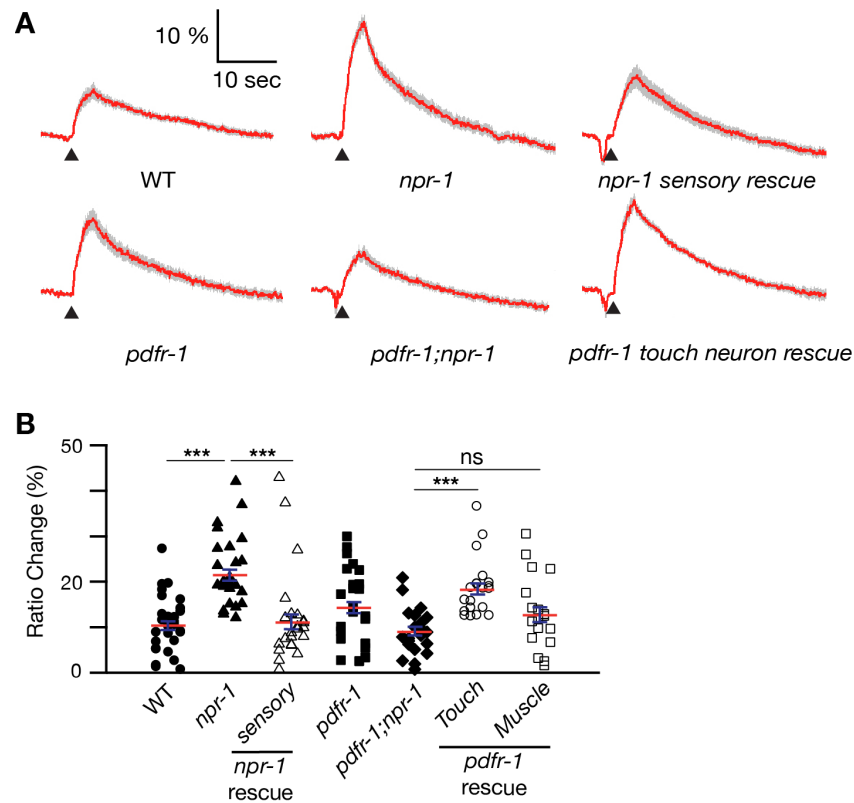


Figure 2.21. PDFR-1 is required for enhanced ALM touch sensitivity in *npr-1* mutant adults.

Touch-evoked calcium transients in ALM were analyzed using cameleon as a calcium indicator. Responses were analyzed in adult animals. Averaged responses (A), and the amplitudes of individual trials (B) are shown for each genotype. Each red trace represents the average percentage change in YFP/CFP fluorescence ratio. The black triangle indicates the time at which the mechanical stimulus was applied. Gray shading indicates SEM of the mean response. (A-B) Touch-evoked calcium transients in adult ALM neurons were significantly larger in *npr-1* mutants. This defect was rescued by transgenes expressing NPR-1 in the RMG circuit (*sensory rescue*), and was suppressed by mutations inactivating PDF-1 and PDFR-1. Touch-evoked calcium transients in *pdfr-1* mutants were not significantly different from wild type controls. Enhanced touch-evoked calcium transients in adult ALM neurons were reinstated in *pdfr-1; npr-1* double mutants by transgenes expressing PDFR-1 in touch neurons but not by those expressed in body muscles. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$; ns, not significant).

magnitude of touch-evoked calcium transients in ALM was significantly increased in *npr-1* mutant adults and this defect was rescued by transgenes expressing NPR-1 in the RMG circuit (Fig. 2.21). The enhanced ALM touch-sensitivity exhibited by *npr-1* adults was eliminated in *pdf-1; npr-1* double mutants (Fig. 2.21) and was reinstated by transgenes expressing PDFR-1 in touch neurons but not by those expressed in body wall muscles (Fig. 2.21). By contrast, in *pdf-1; npr-1* double mutants, heightened ALM touch responsiveness was reduced but not eliminated (Fig. 2.22). The residual effect of NPR-1 on ALM touch sensitivity in *pdf-1; npr-1* double mutants was likely mediated by other

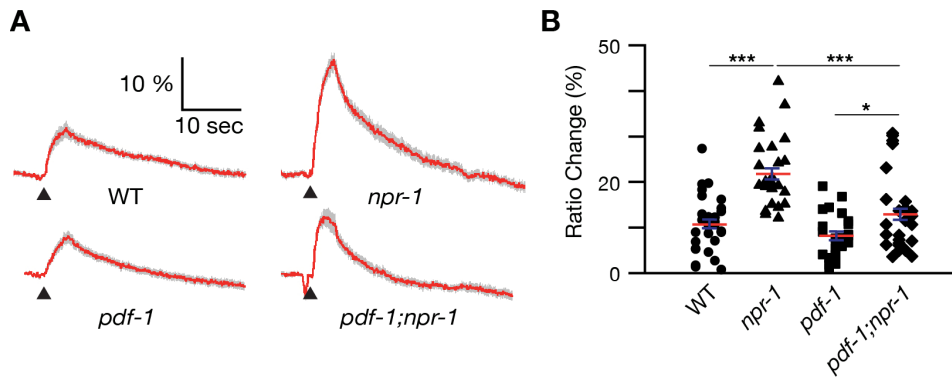


Figure 2.22. PDF-1 is required for enhanced ALM touch sensitivity in *npr-1* mutant adults.

Touch-evoked calcium transients in adult ALM neurons were analyzed using cameleon as a calcium indicator. Averaged responses (A) and the amplitudes of individual trials (B) are shown for each genotype. Each red trace represents the average percentage change in YFP/CFP fluorescence ratio. The black triangle indicates the time at which the mechanical stimulus was applied. Gray shading indicates SEM of the mean response. The heightened touch-evoked calcium transient observed in *npr-1* mutants was reduced but not eliminated in *pdf-1; npr-1* double mutants. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; ***, $p < 0.001$).

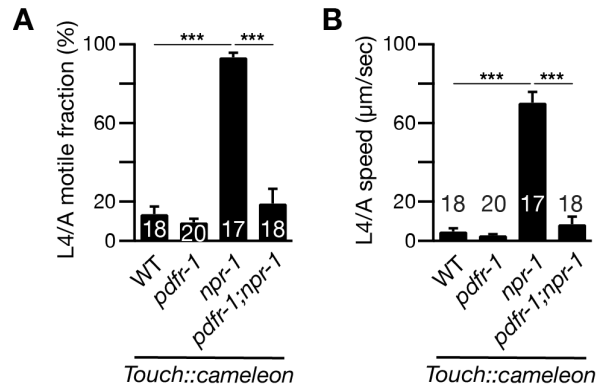


Figure 2.23. Cameleon expression in touch neurons does not disrupt NPR-1 and PDFR-1 effects on L4/A locomotion quiescence

Locomotion behavior of single worms carrying cameleon expressed in touch neurons was analyzed during the L4/A lethargus in the indicated genotypes. Average motile fraction (A), and average locomotion velocity (B) are plotted. Cameleon expression in touch neurons did not disrupt NPR-1 and PDFR-1 effects on L4/A locomotion quiescence. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

PDFR-1 ligands (e.g. PDF-2). Collectively, these results suggest that increased PDF-1 secretion in *npr-1* adults was associated with enhanced touch sensitivity.

Because PDF-1 and PDFR-1 enhanced touch sensitivity in *npr-1* mutants, we would expect that *pdf-1* and *pdf-1* single mutants would exhibit decreased touch sensitivity. Contrary to this idea, adult ALM touch responses were unchanged in either single mutant (Fig. 2.21 and 2.22). These results do not exclude the idea that touch sensitivity was altered in these mutants. We may fail to detect differences in ALM responses for technical reasons. For example, an effect on touch sensitivity in single mutants may only be apparent at lower stimulus intensities, or upon repetitive stimulation. To further address this issue, we analyzed locomotion in the single mutants.

Adult *pdf-1* and *pdf-1* single mutants exhibited significantly slower locomotion and decreased motile fractions (Fig. 2.24) (Meelkop et al., 2012), both of which could result from diminished touch sensitivity. Consistent with this idea, the decreased locomotion rate and motile fraction of *pdf-1* mutants was partially rescued by transgenes expressing PDFR-1 in touch neurons (Fig. 2.24). These results support the idea that PDF-1 and PDFR-1's effects on touch sensitivity are not restricted to *npr-1* mutants.

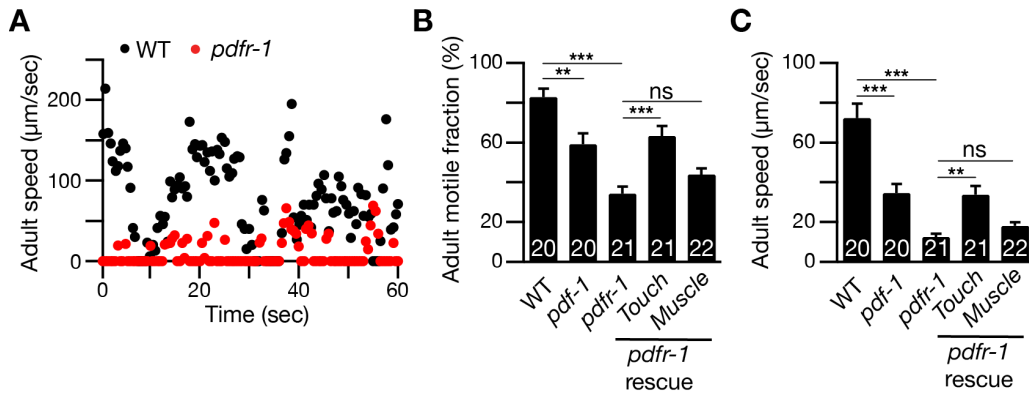


Figure 2.24. The *pdf-1* adult locomotion defect is partially due to altered touch sensitivity.

Locomotion behavior of single adult worms was analyzed in the indicated genotypes. Instantaneous locomotion velocity (A), average motile fraction (B), and average locomotion velocity (C) are plotted. Both *pdf-1* and *pdf-1* single mutants showed reduced locomotion in adult. The *pdf-1* adult locomotion defect was partially rescued by transgenes expressing PDFR-1 in touch neurons, but not in body wall muscles. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$; **, $p < 0.01$; ns, not significant).

To determine if NPR-1 also regulates touch sensitivity during lethargus, we analyzed ALM calcium transients during the L4/A lethargus (Fig. 2.25). A recent study reported that touch neuron calcium transients are significantly reduced during lethargus

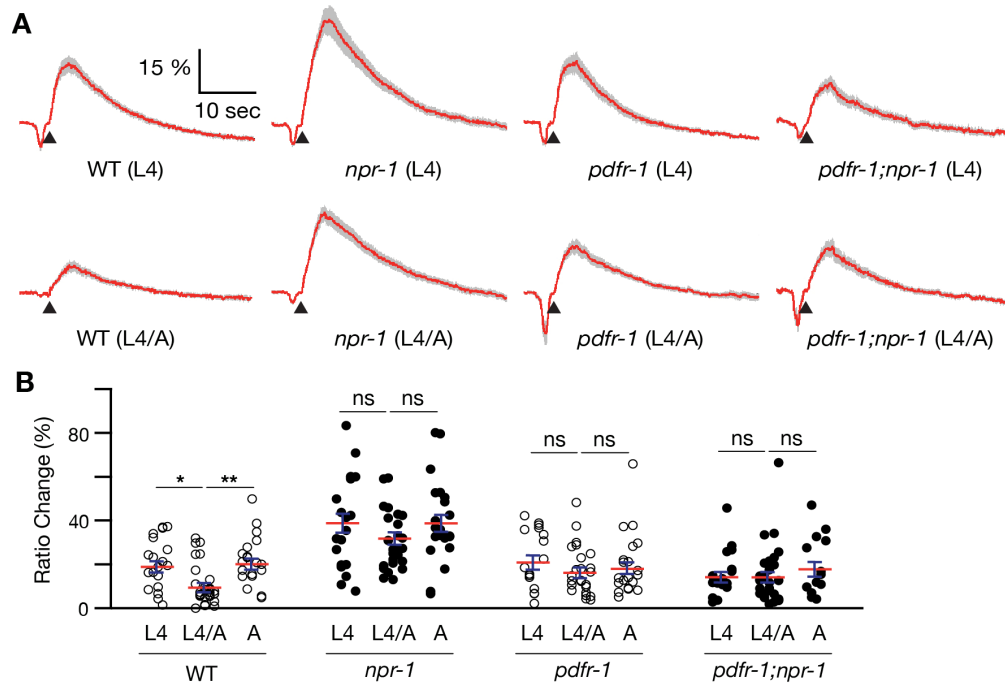


Figure 2.25. NPR-1 and PDFR-1 regulate ALM touch sensitivity during lethargus.

Touch-evoked calcium transients in ALM were analyzed in L4, L4/A, and adults of the indicated genotypes. Averaged responses (A) and the amplitudes of individual trials (B) are shown for each genotype. Each red trace represents the average percentage change in YFP/CFP fluorescence ratio. The black triangle indicates the time at which the mechanical stimulus was applied. Gray shading indicates SEM of the mean response. (A-B) Touch-evoked ALM calcium transients were significantly reduced during L4/A lethargus, and this effect was abolished in *npr-1* mutants. Enhanced touch-evoked calcium transients in *npr-1* mutants were suppressed by inactivating PDFR-1. Values that differ significantly are indicated (*, $p < 0.05$; **, $p < 0.01$; ns, not significant).

(Schwarz et al., 2011). Consistent with this prior study, we found that ALM touch-evoked calcium transients were significantly smaller during the L4/A lethargus; however, this effect was eliminated in *npr-1* mutants (Fig. 2.25). The enhanced ALM touch responses during lethargus exhibited by *npr-1* mutants was eliminated in *pdfr-1; npr-1*

double mutants (Fig. 2.25). Thus, NPR-1 inhibition of PDF signaling is required for inhibition of touch sensitivity during lethargus.

DISCUSSION

We describe a circuit mechanism controlling arousal from a developmentally programmed form of behavioral quiescence in *C. elegans*. Increased RMG circuit activity in *npr-1* mutants was accompanied by increased PDF-1 secretion and heightened peripheral sensitivity to touch, thereby increasing motility during lethargus. Below we discuss the significance of these results.

Related neuropeptides mediate quiescence and arousal/motivation in worms, flies, and rodents. Peptides homologous to NPY induce locomotion quiescence in *C. elegans* (FLP-18 and -21), inhibit locomotion and foraging on food in *Drosophila* (NPF) (Wu et al., 2003), and inhibit the arousing effects of hypocretin expressing neurons in mice (NPY) (Fu et al., 2004). By contrast, peptides homologous to PDF arouse locomotion in *C. elegans* (PDF-1), arouse circadian locomotor activity and decrease sleep duration in *Drosophila* (PDF) (Parisky et al., 2008; Renn et al., 1999), and regulate circadian behaviors and sleep in rodents (VIP) (Hu et al., 2011a; Maywood et al., 2007). Thus, conserved molecular mechanisms are employed to regulate arousal and quiescence in developmentally programmed, metabolically driven, and circadian behavioral states.

If lethargus is a sleep-like state, as previously proposed (Raizen et al., 2008; Van Buskirk and Sternberg, 2007), one would expect that disrupting quiescence during lethargus would be deleterious. Contrary to this notion, the fertility and development of *npr-1* mutants were not grossly altered, indicating that locomotion quiescence during lethargus is not essential for normal development or molting. These results do not

exclude the idea that quiescence during lethargus has significant effects on health in native environments (where conditions are more variable).

How are arousal peptides functionally coupled to circadian and developmental cycles? VIP and PDF are expressed in central clock neurons: rat VIP in the suprachiasmatic nucleus (SCN) of the hypothalamus, fly PDF in LNV neurons, and worm PDF in the RMG circuit (Helfrich-Forster, 1995; Maywood et al., 2007). Rhythmic changes in *pdf* mRNA levels were not observed in the *Drosophila* circadian and *C. elegans* molting cycles (Janssen et al., 2009; Park and Hall, 1998). Instead, PDF-1 secretion was dramatically reduced during lethargus. Inhibition of PDF-1 secretion and inhibition of locomotion during lethargus were both abolished in *npr-1* mutants. Thus, altered PDF-1 secretion provides a cellular mechanism for coupling changes in locomotor activity to the molting cycle.

How is PDF-1 secretion inhibited during lethargus? In *npr-1* mutants, pheromone, and oxygen responses mediated by the RMG circuit are enhanced (Cheung et al., 2005; Gray et al., 2004; Macosko et al., 2009) and we observed a corresponding enhancement of PDF-1 secretion. Similarly, inactivation and restoration of TAX-4 CNG channel expression in the RMG circuit was accompanied by parallel changes in PDF-1 secretion. Based on these results, we propose that RMG circuit activity is diminished during lethargus, thereby inhibiting PDF-1 secretion. Consistent with this idea, forced depolarization of ASH neurons expressing PDF-1 was sufficient to arouse locomotion during lethargus.

How do central clock neurons engender rhythmic behaviors? A great deal is known about how the activity and expression profile of central clock neurons are

regulated. Much less is known about how clock neurons dictate circadian behaviors. In *C. elegans*, responsiveness to several sensory cues is reduced during lethargus. In particular, touch sensitivity and touch-evoked calcium transients in the touch neurons are decreased during lethargus (Raizen et al., 2008; Schwarz et al., 2011; Singh et al., 2011). Our results provide a cellular mechanism for these effects. During lethargus, NPR-1 inhibited PDF-1 secretion from the RMG circuit, thereby decreasing touch neuron sensitivity. PDF-1's effect on locomotion arousal was also mediated in part by activation of PDFR-1 receptors in body muscle. Interestingly, fly PDF and rodent VIP also have direct effects on muscle function (Talsma et al., 2012).

Although NPR-1, TAX-4, and PDF have profound effects on lethargus behavior, several results suggest that other signaling pathways must also contribute to both quiescence and arousal. For example, L4/A quiescence was restored in *pdf-1; npr-1* double mutants (Fig. 3D-F); consequently, changes in NPR-1 and PDF signaling are not absolutely required to induce locomotion quiescence or arousal. Similarly, the locomotion of *pdf-1* mutants during lethargus was significantly more quiescent than in adults. Thus, inactivating PDF signaling is unlikely to be the only mechanism producing L4/A quiescence. These results suggest that arousal and quiescence are behavioral states governed by multiple inputs, whose activities are integrated in the RMG circuit.

NPR-1 regulates several physiologically important traits. Inactivating NPR-1 alters sensitivity to environmental repellents (e.g. pheromones and oxygen), foraging behavior, innate immune responses, and lethargus behavior (Cheung et al., 2005; de Bono and Bargmann, 1998; Gray et al., 2004; Reddy et al., 2009; Styer et al., 2008) (Fig. 2A). Because NPR-1 sits at the nexus of multiple physiologically important traits, changes in

NPR-1 activity and natural variation in the *npr-1* gene provide a mechanism for coupling changes in behavioral quiescence to the demands of the local environment. Specifically, changes in NPR-1 signaling could allow isolated populations to optimize growth properties in environments with increased exposure to specific repellents or bacterial pathogens.

MATERIALS AND METHODS

Strains

Strain maintenance and genetic manipulation were performed as described (Brenner, 1974). Animals were cultivated at 20°C on agar nematode growth media seeded with OP50 *E.coli*. Wild type reference strain was N2 Bristol. Strains used in this study are as follows:

Wild type strains

CB4555, TR389, AB3, CB4856, and RC301

Mutant strains and integrants

DA609 *npr-1(ad609) X*

KP6080 *npr-1(g320) X*

KP6048 *npr-1(ky13) X*

AX1410 *flp-18(db99) X* (Gift from Mario de Bono)

KP6077 *flp-21(pk1601) V*

PR678 *tax-4(p678) III*

KP3183 *osm-9(ky10) IV*

KP5966 *egl-3(nr2090) V*

KP5989 *pkc-1(nj3) V*

LSC27 *pdf-1(tm1996) III*

KP6340 *pdf-1(ok3425) III*

KP6416 *pdf-2(tm4393) X*

CB1338 *mec-3(e1338) IV*

KP7044 *flp-21(pk1601) V;flp-18(db99) X*
 KP7041 *flp-18(db99) npr-1(g320) X*
 KP7042 *flp-21(pk1601) V;npr-1(g320) X*
 KP7059 *flp-21(pk1601) V;flp-18(db99) npr-1(g320) X*
 KP6060 *tax-4(p678) III;npr-1(ky13) X*
 KP6841 *osm-9(ky10) IV;npr-1(ky13) X*
 KP6054 *egl-3(nr2090) V;npr-1(ky13) X*
 KP6682 *pkc-1(nj3) V;npr-1(ky13) X*
 KP6100 *pdf-1(tm1996) III;npr-1(ky13) X*
 KP6410 *pdf-1(ok3425) III;npr-1(ky13) X*
 KP6417 *pdf-2(tm4393) npr-1(ky13) X*
 KP5364 *nre-1(hd20) lin-15b(hd126) X*
 KP6050 *npr-1(ky13) nre-1(hd20) lin15b(hd126) X*
 CX4978 *kyIS200[sra-6p::VR1, elt-2p::NLS-gfp]* (Gift from Cori Bargmann)
 KP6426 *mec-3(e1338) IV;npr-1 (ky13) X*
 KP6693 *nuIS472[pdf-1p::pdf-1::YFP, vha-6p::mCherry]*
 KP6744 *tax-4(p678) III;nuIS472*
 KP6745 *tax-4(p678) III;npr-1(ky13) X;nuIS472*
 KP6743 *npr-1(ky13) X;nuIS472*
 AQ906 *bzIS17[mec-4p::YC2.12]*
 KP6679 *pdf-1(ok3425) III;bzIS17*
 KP6680 *pdf-1(ok3425) III;npr-1(ky13) X;bzIS17*
 KP6681 *npr-1(ky13) X;bzIS17*

KP6699 *pdf-1(tm1996) III;npr-1(ky13) X;bzIS17*

KP6700 *pdf-1(tm1996) III; bzIS17*

Strains containing extrachromosomal arrays

CX9396 *npr-1(ad609) X;kyEX1966[flp-21p::npr-1 SL2 GFP, ofm-1p::dsRed]* (Gift from Cori Bargmann)

KP6053 *npr-1(ad609) X;nuEX1520[unc-30p::npr-1::gfp, myo-2p::NLS-mCherry]*

KP7144 *tax-4(p678) III;npr-1(ky13) X;nuEX1601[flp-21p::tax-4, vha-6p::mCherry]*

KP7141 *npr-1(ky13) X;nuIS472;nuEX1607[flp-21p::npr-1, myo-2p::NLS-mCherry]*

KP7143 *tax-4(p678) III;npr-1(ky13) X;nuIS472;nuEX1612[flp-21p::tax-4, myo-2p::NLS-mCherry]*

KP6819 *nuEX1560[unc-17p::rig-3(-GPI)::mCherry]*

KP6820 *npr-1(ky13) X; nuEX1560*

KP7053 *kyIS200;nuEX1610[sra-6p::pdf-1::venus, myo-2p::NLS-mCherry]*

KP6678 *pdf-1(tm1996) III;npr-1(ky13) X;nuEX1547[sra-9p::pdf-1::venus]*

KP6741 *pdf-1(tm1996) III;npr-1(ky13) X;nuEX1552[str-3p::pdf-1::venus, vha-6p::mCherry]*

KP6860 *nuEX1611[sra-9p::pdf-1::venus, myo-2p::NLS-mCherry]*

KP7146 *npr-1(ky13);nuEX1611*

KP6423 *pdf-1(ok3425) III;npr-1(ky13) X;nuEX1526[mec-3p::pdf-1b, myo-2p::NLS-mCherry]*

KP6594 *pdf-1(ok3425) III;npr-1(ky13) X;nuEX1534[myo-3p::pdf-1a, vha-6p::mCherry]*

KP6733 *pdfr-1(ok3425) III;npr-1(ky13) X;bzIS17; nuEX1526 [mec-3p::pdfr-1b, myo-2p::NLS-mCherry]*

KP6734 *pdfr-1(ok3425) III;npr-1(ky13) X;bzIS17;nuEX1534[myo-3p::pdfr-1a, vha-6p::mCherry]*

KP6736 *npr-1(ad609) X;bzIS17; kyEX1966[flp-21p::npr-1 SL2 GFP, ofm-1p::dsRed]*

KP6815 *pdfr-1(ok3425) III;nuEX1526[mec-3p::pdfr-1b, myo-2p::NLS-mCherry]*

KP6816 *pdfr-1(ok3425) III;nuEX1534[myo-3p::pdfr-1a, vha-6p::mCherry]*

Constructs

***pdf-1* expression constructs (*pdf-1p::pdf-1::YFP* (KP#1861), *sra-9p::pdf-1::YFP* (KP#1923), *str-3p::pdf-1::YFP* (KP#1924), and *sra-6p::pdf-1::YFP* (KP#1925))**

cDNAs corresponding to *pdf-1* and YFP (VENUS) containing a stop codon were each amplified by PCR and ligated into pPD49.26 (Addgene) containing the *pdf-1* (~5.4kb 5' regulatory sequence), *sra-9* (~3kb 5' regulatory sequence: ASK expression), *str-3* (~3kb 5' regulatory sequence: ASI expression), and *sra-6* (~3.8kb 5' regulatory sequence: ASH expression) promoters.

***npr-1* rescue constructs (*unc-30p::npr-1::GFP* (KP#1857) and *flp-21p::npr-1* (KP#1921))**

npr-1 cDNA (215V) was amplified by PCR and ligated into expression vectors (pPD49.26) containing the *unc-30* promoter (~2.5kb 5' regulatory sequence) and GFP at the 3' end of MCSII or the *flp-21* promoter (~4.1kb 5' regulatory sequence).

***tax-4* rescue construct (*flp-21p::tax-4* (KP#1922))**

tax-4 cDNA was amplified by PCR and ligated into expression vector (pPD49.26) containing the *flp-21* promoter (~4.1kb 5' regulatory sequence).

***pdf-1* rescue constructs (*mec-3p::pdf-1b* (KP#1863) and *myo-3p::pdf-1a* (KP#1866))**

pdf-1 cDNAs were amplified by PCR and ligated into expression vectors (pPD49.26) containing the *mec-3* promoter (3.4kb upstream of the start codon of *mec-3* genomic region) or *myo-3* promoter (~2.4kb 5' regulatory sequence).

Transgenes and germline transformation

Transgenic strains were generated by microinjection of various plasmids with coinjection markers (*myo-2p::NLS-mCherry* (KP#1480) and *vha-6p::mcherry* (KP#1874)). Injection concentration was 40 - 50 ng/μl for all the expression constructs and 10 ng/μl for coinjection markers. The empty vector *pBluescript* was used to bring the final DNA concentration to 100 ng/μl. Integration of transgenes was obtained by UV irradiation of strains carrying extrachromosomal arrays. All the integrants were outcrossed to wild type strains (N2 bristol) 10 times.

Lethargus locomotion and behavior analysis

Well-fed late L4 animals were transferred to full lawn OP50 bacterial plates. After 1 hour, locomotion of animals in lethargus (determined by absence of pharyngeal pumping) was recorded on a Zeiss Discovery Stereomicroscope using Axiovision software.

Locomotion was recorded at 2 Hz for 30-75 seconds. Centroid velocity of each animal was analyzed at each frame using object-tracking software in Axiovision. Motile fraction

of each animal was calculated by dividing the number of frames with positive velocity value with total number of frames. Speed of each animal was calculated by averaging the velocity value at each frame. For long-term lethargus locomotion analysis (Fig. S1A-B), 1 min-long video was recorded every 20 minutes for each animal after the transfer to full lawn OP50 bacterial plates, and motile fraction was calculated for each time point. For the forced secretion of PDF-1 (Fig. 4C-D), early L4 animals were transferred to NGM plates containing 50 μ M capsaicin (with food) and treated with capsaicin for 6-7 hours. Duration of L4/A pumping quiescence was calculated by summing the time period from the cessation to the resumption of pharyngeal pumping. Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparison and two-tailed Student's t test for pairwise comparison.

Adult locomotion and behavior analysis

Locomotion of adult animals was analyzed with the same setup as lethargus locomotion analysis described above, except that well-fed adult animals were monitored within 5-10 minutes after the transfer to full lawn OP50 bacterial plates. Pharyngeal pumping rate of adult animals was calculated by counting the number of pharyngeal pumping for 10 seconds under the Leica MS5 routine stereomicroscope. Foraging behavior was analyzed as described (de Bono and Bargmann, 1998). Briefly, approximately 150 well-fed adult animals were placed on NGM plates seeded with 200 μ l OP50 *E.coli* 2 days before the assay. After 3 hours, images were taken for each genotype. Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparison and two-tailed Student's t test for pairwise comparison.

RNAi feeding screen

A small-scale RNAi feeding screen was performed as described (Kamath et al., 2003). The screen was performed in the neuronal RNAi hypersensitive mutant background (*nre-1 lin-15b*) (Schmitz et al., 2007). 15 neuropeptide genes known to be expressed in RMG circuit were selected for the screen (Li and Kim, 2008). After 5 days of RNAi treatment (2 generation) at 20°C, well-fed late L4 animals were transferred to full lawn OP50 bacterial plates. After 1 hour, animals in lethargus (determined by absence of pharyngeal pumping) were scored for their motility. Statistical significance was determined using chi-square test.

Quantitative PCR

Total RNA was purified from synchronized animals in L4/A lethargus (determined by absence of pharyngeal pumping) and synchronized young adult animals (4-5 hours after L4/A lethargus) using standard protocol. 6 biological replicates of wild type (N2 Bristol) and *npr-1(ky13)* samples were collected on 3 different days. 2 µg of total RNA was used to synthesize cDNA using RETROscript (Ambion). Real-time PCR was performed using iTaq SYBR Green Supermix with ROX (BioRad) and a 7500 Fast Real-Time PCR System (Applied Biosystems). Statistical significance was determined using two-tailed Student's t test.

Fluorescence microscopy and image analysis

Quantitative imaging of coelomocyte fluorescence was performed using a Zeiss Axioskop equipped with an Olympus PlanAPO 100x (NA=1.4) objective and a

CoolSNAP HQ CCD camera (Photometrics). Worms were immobilized with 30 mg/ml BDM (Sigma). The anterior coelomocytes were imaged in L4, L4/A lethargus (determined by absence of pharyngeal pumping), young adult (0-2 eggs), and gravid adult animals. Image stacks were captured and maximum intensity projections were obtained using Metamorph 7.1 software (Universal Imaging). YFP fluorescence was normalized to the absolute mean fluorescence of 0.5 mm FluoSphere beads (Molecular Probes). Statistical significance was determined using one-way ANOVA with Tukey test.

Calcium imaging and analysis

To image touch-evoked calcium transients in the ALM cell body, we used a transgenic line (*bzIs17*) that expresses the calcium-sensitive protein cameleon in touch neurons (using the *mec-4* promoter). Calcium imaging was performed on a Zeiss Axioskop 2 upright compound microscope equipped with a Dual View Beam Splitter and a Uniblitz Shutter. Images were recorded at 10 Hz using an iXon EM camera (Andor Technology) and captured using IQ1.9 software (Andor Technology). Individual worms were glued using Dermabond topical skin adhesive glue to pads composed of 2% agarose in extracellular saline (145mM NaCl, 5mM KCl, 1mM CaCl₂, 5mM MgCl₂, 20mM D-glucose, 10mM HEPES buffer, pH7.2). Gentle touch stimuli were delivered using a M-111.1DG micromanipulator. The micromanipulator was used to drive a pulled glass microcapillary with a 15µm diameter rounded tip against the side of the glued worm. The tip was positioned adjacent to the body wall and was driven forward to cause a 10µm (adults, Fig. 6) or 20µm (L4/A lethargus, Fig. 7) deflection of the cuticle. Optical and mechanical stimuli were synchronized by flashing a white LED on the sample a second

before the stimulus was delivered. Analysis was done using a custom written Matlab (Mathworks) program. A rectangular region of interest (ROI) was drawn surrounding the cell body and for every frame the ROI was shifted according to the new position of the center of mass. Fluorescence intensity, F , was computed as the difference between the sum of pixel intensities and the faintest 10% pixels (background) within the ROI. Statistical significance was determined using one-way ANOVA with Tukey test.

Chapter 3

NPR-1 Regulates Excitatory Synaptic Transmission at *C. elegans* Neuromuscular Junctions

The experiments discussed in this chapter are unpublished work resulting from collaboration between Seungwon Choi and Zhitao.

Seungwon Choi learned electrophysiological recordings on body muscles of dissected worms under the guidance of two postdocs in Joshua Kaplan lab, Edward Pym and Zhitao Hu. Seungwon Choi and Zhitao Hu equally contributed to electrophysiology data in this chapter. Seungwon Choi performed electrophysiological recordings presented in Fig 3.1, Fig 3.3, Fig 3.6, and Fig 3.10, while Zhitao Hu performed electrophysiological recordings presented in Fig 3.1, Fig 3.4, Fig 3.5, and Fig 3.9. Seungwon Choi performed all of the other experiments.

INTRODUCTION

Animals perceive diverse sensory stimuli from environment throughout life. Sensory experience plays a key role in the proper development and function of nervous system. The perturbation of sensory perception not only impairs cognitive function by disrupting normal development of relevant neural circuits, but also prevents animals from properly responding to environmental cues and adapting to new environmental conditions, endangering health and survival of animals.

Sensory experience is required for both structural and functional plasticity of neural circuits. A great deal is known about sensory experience-dependent structural change in neural circuits. For example, monocular deprivation during critical period shifts ocular dominance columns in the visual cortex in mammals (Hensch, 2005; Wiesel and Hubel, 1963). Similarly, whisker trimming alters the turnover of dendritic spines in mouse barrel cortex (Holtmaat and Svoboda, 2009). However, relatively little is known about sensory experience-dependent changes in electrophysiological properties at individual synapses in relevant neural circuits. To address this question, we analyzed sensory-evoked change in synaptic transmission at *C. elegans* neuromuscular junctions (NMJs).

The *C. elegans* nervous system consists of 302 neurons that develop a stereotyped pattern of synaptic contacts. 32 sensory neurons receive sensory inputs from environment and make synaptic connections with downstream interneurons. Among those, command interneurons innervate cholinergic motor neurons along the ventral cord of worms, which in turn form dyadic synaptic contacts with both GABAergic motor neurons and body wall

muscles. At *C. elegans* NMJs, body muscles receive two distinct synaptic inputs, excitatory inputs mediated by acetylcholine (ACh) and inhibitory inputs mediated by GABA.

Synaptic activity in *C. elegans* body muscles can be assayed by measuring sensitivity to acetylcholine esterase inhibitor aldicarb. Aldicarb treatment causes accumulation of ACh at NMJs, leading to paralysis of worms. The time course of paralysis shifts depending on the synaptic strength in body muscles. Decreased ACh release confers to aldicarb resistance, whereas increased ACh release confers to aldicarb hypersensitivity (Gracheva et al., 2006; McEwen et al., 2006). By contrast, decreased GABA release confers to aldicarb hypersensitivity, whereas increased GABA release confers to aldicarb resistance (Loria et al., 2004). Systematic RNAi screens for aldicarb resistance and hypersensitivity have identified many genes that are required for general synaptic transmission as well as genes that specifically regulate excitatory or inhibitory synapses (Sieburth et al., 2005; Vashlishan et al., 2008).

Another and more direct approach to assay synaptic activity at *C. elegans* NMJs is to measure postsynaptic currents in body muscles using electrophysiological recordings (Richmond et al., 1999). Live worms can be dissected in a way that allows access to NMJs and patch-clamp recordings on muscle cells. Excitatory ACh-gated currents and inhibitory GABA-gated currents can be separated from each other by holding muscle cells at reversal potential of GABA-gated currents (-60 mV) and ACh-gated currents (0 mV), respectively. In this way, one can distinguish cholinergic and GABAergic contribution to body muscle activity.

Although a great deal is known about the molecular mechanism controlling synaptic activity in local neural circuits, relatively little is known about how sensory inputs modulate synaptic activity in the neural circuits located at a distance from sensory organs, and what signaling entities mediate this sensory-evoked change in synaptic activity. Here we identify a central sensory circuit that regulates cholinergic transmission at *C. elegans* NMJs. Increased sensory activity in the central circuit is associated with enhancement of endogenous EPSC rate at NMJs. This central circuit regulates cholinergic transmission in body muscles through the concerted action of glutamate and neuropeptide. These results provide a circuit mechanism controlling sensory-evoked enhancement of cholinergic synapses in *C. elegans*.

RESULTS

Endogenous excitatory synaptic transmission is increased in *npr-1* mutants

In the previous RNAi screen, *npr-1* was identified whose knockdown caused aldicarb hypersensitivity (Vashlishan et al., 2008). Aldicarb sensitivity is assayed by monitoring the time course of paralysis of a population of animals during a 2 hour aldicarb treatment. We first repeated aldicarb assay with *npr-1* mutants, and confirmed *npr-1* mutants are hypersensitive to aldicarb as previously reported (Fig. 3.1A). Based on electrophysiological recordings that revealed decreased endogenous IPSCs (synaptic events mediated by the endogenous activity of GABAergic motor neurons) in *npr-1* mutants, it was originally suggested that aldicarb hypersensitivity of *npr-1* mutants is caused by decreased GABA transmission at NMJs (Vashlishan et al., 2008). However, only a small number of *npr-1* mutant animals were analyzed in the previous study, requiring more extensive electrophysiological recordings on *npr-1* mutants.

We recorded both EPSCs evoked by a depolarizing stimulus and endogenous EPSCs (synaptic events mediated by the endogenous activity of cholinergic motor neurons) from adult body wall muscles. The endogenous EPSC rate was greatly increased, and the amplitude was also slightly increased in *npr-1* mutants (Fig. 3.1D-F). By contrast, the amplitude of evoked EPSCs (Fig. 3.1B-C), the rate and the amplitude of endogenous IPSCs were unaltered in *npr-1* mutants (Fig. 3.1G-I), suggesting that endogenous cholinergic transmission is specifically enhanced in *npr-1* mutants. The density of SNB-1 (synaptobrevin) puncta in cholinergic axons was normal in *npr-1*

mutants, suggesting that the increased cholinergic transmission was not caused by increase in the number of cholinergic NMJs (Vashlishan et al., 2008).

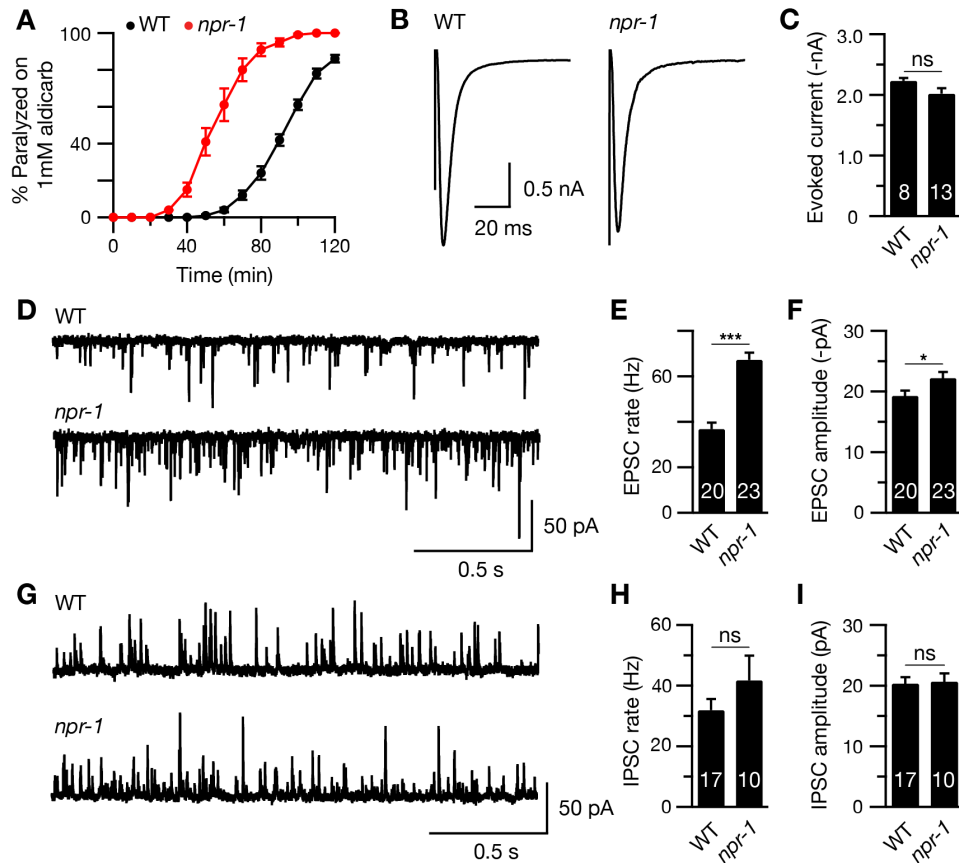


Figure 3.1. NPR-1 regulates endogenous cholinergic transmission at NMJs.

(A) Time course of paralysis for *npr-1(ky13)* mutant versus wild-type animals on 1 mM aldicarb (n=13 and 15 trials, respectively, and 20-30 worms per trial). *npr-1* mutants were hypersensitive to aldicarb. (B-I) Stimulus-evoked EPSCs (B, C), endogenous EPSCs (D-F), and endogenous IPSCs (G-I) were recorded from body wall muscles of the adult worms for the indicated genotypes. Averaged traces of stimulus-evoked EPSCs (B), representative traces of endogenous EPSCs (D) and IPSCs (G), and summary data are shown (C, E, F, H, and I). Endogenous EPSCs were increased in *npr-1* mutants (D-F), whereas stimulus-evoked EPSCs and endogenous IPSCs were normal (B, C, G, H, and I). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; ***, $p < 0.001$; ns, not significant).

The NPR-1 ligands FLP-21 and FLP-18 have no effect on aldicarb sensitivity

Two NPR-1 ligands, FLP-21 and FLP-18, have been identified in the screen for neuropeptides that are capable of activating NPR-1 in *Xenopus* oocytes, *C. elegans* pharyngeal muscles, and heterologous cells (Kubiak et al., 2003; Rogers et al., 2003). To determine if these two neuropeptides function as NPR-1 ligands and regulate cholinergic transmission, we performed aldicarb assays with *flp-18* and *flp-21* mutants. Both *flp-18* and *flp-21* single mutants showed normal sensitivity to aldicarb (Fig. 3.2A). To exclude the possibility of functional redundancy between FLP-18 and FLP-21, *flp-21;flp-18* double mutants were also analyzed. Like single mutants, *flp-21;flp-18* double mutants had no significant alteration in aldicarb sensitivity (Fig. 3.2A), suggesting that these two neuropeptides can not activate NPR-1, at least, in worms expressing high affinity NPR-1(215V) receptors (N2 Bristol) (Kubiak et al., 2003; Rogers et al., 2003). However, as in other *npr-1* phenotype such as social foraging (Rogers et al., 2003) and lethargus

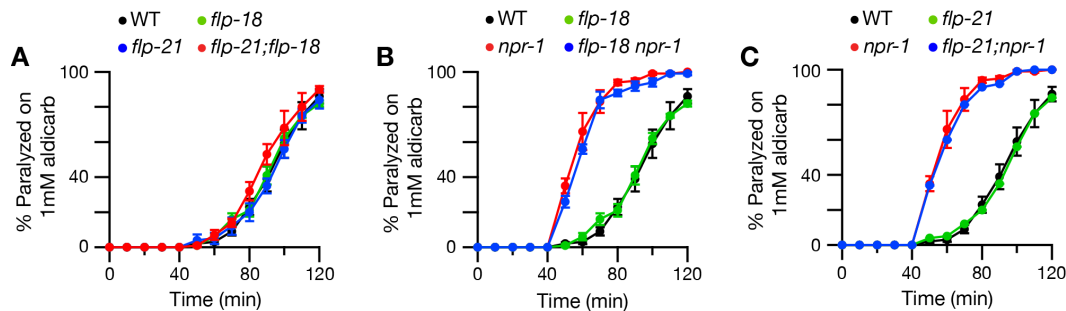


Figure 3.2. The NPR-1 ligands FLP-21 and FLP-18 have no effect on aldicarb sensitivity.

(A-C) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=7, 5, 5, 5, 5, 4, and 4 trials for wild-type, *flp-18(tm2179)*, *flp-21(pk1601)*, *npr-1(ky13)*, *flp-21;flp-18*, *flp-18 npr-1*, and *flp-21;npr-1* animals, respectively, and 20-30 worms per trial). Mutations inactivating FLP-18 and FLP-21 had no effect on aldicarb sensitivity.

behavior (Fig. 2.2), there remains a possibility that inactivating FLP-18 and/or FLP-21 may affect aldicarb sensitivity in a Bristol strain expressing low affinity NPR-1(215F) receptors (*npr-1(g320)* mutants). This possibility should be further explored by examining *flp-21;npr-1(g320)* and *flp-18 npr-1(g320)* double mutants. As a control, we first analyzed aldicarb sensitivity of *flp-21;npr-1(null)* and *flp-18 npr-1(null)* double mutants, and confirmed that inactivating neither FLP-21 nor FLP-18 affected aldicarb-induced paralysis of *npr-1* null animals (Fig. 3.2B-C).

The *npr-1* cholinergic transmission defect is mediated by increased sensory activity

NPR-1's effects on foraging (Macosko et al., 2009) and lethargus behavior (Fig. 2.2) are mediated by its expression in the RMG circuit in the head. In addition to the RMG circuit, NPR-1 is also expressed in GABAergic motor neurons in the ventral nerve cord (Coates and de Bono, 2002). We did two experiments to determine where NPR-1 functions to regulate cholinergic transmission at NMJs. First, an *npr-1* transgene expressed in the RMG circuit (using the *flp-21* promoter) completely rescued both aldicarb hypersensitivity and cholinergic transmission defect of *npr-1* mutants, whereas transgenes expressed in GABAergic motor neurons (using the *unc-30*, a gene encoding homeodomain protein involved in the differentiation of GABAergic motor neurons, and *unc-25*, a gene encoding glutamic acid decarboxylase (GAD), promoters) had no rescuing activity (Fig. 3.3). Interestingly, transgenes expressed in GABAergic motor neurons made *npr-1* mutants more hypersensitive to aldicarb than *npr-1* mutants without the transgenes (Fig. 3.3A), which is likely caused by NPR-1's inhibition of GABA

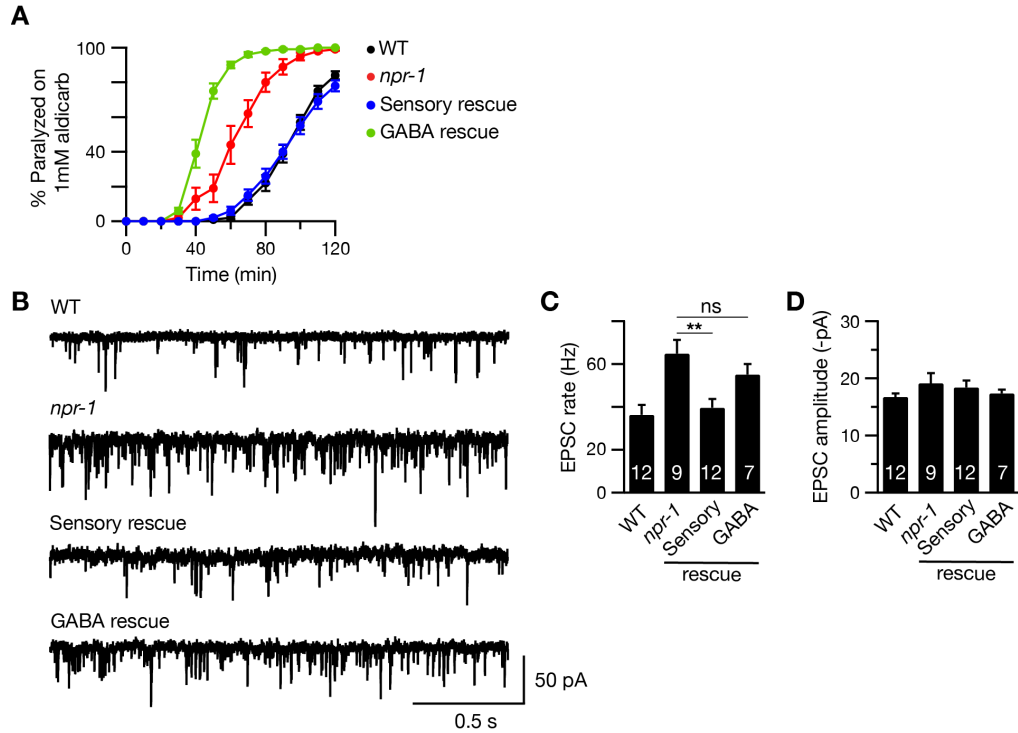


Figure 3.3. NPR-1 expressed in the RMG circuit regulates endogenous cholinergic transmission at NMJs.

(A) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes ($n=11$, 6, 8, and 15 trials for wild-type, *npr-1(ad609)*, Sensory rescue, and GABA rescue, respectively, and 20-30 worms per trial). *npr-1* aldicarb hypersensitivity was rescued by transgenes expressing NPR-1 in the RMG circuit (Sensory rescue, *flp-21* promoter) but not by those expressed in GABAergic neurons (GABA rescue, *unc-25* and *unc-30* promoters), using the indicated promoters. (B-D) Endogenous EPSCs were recorded from body wall muscles of the adult worms for the indicated genotypes. Representative traces of endogenous EPSCs (B) and summary data are shown (C, D). The *npr-1* cholinergic transmission defect was rescued by transgenes expressing NPR-1 in the RMG circuit (Sensory rescue) but not by those expressed in GABAergic neurons (GABA rescue). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (**, $p < 0.01$; ns, not significant).

transmission through G_o signaling pathway (Miller et al., 1999; Nurrish et al., 1999). Second, both aldicarb hypersensitivity and cholinergic transmission defect of *npr-1* mutants were abolished by mutations inactivating ion channels required for sensory transduction, such as TAX-4/CNG, OSM-9 and OCR-2/TRPV channels (Figs. 3.4 and 3.5). Together, these results suggest that cholinergic transmission defect in *npr-1*

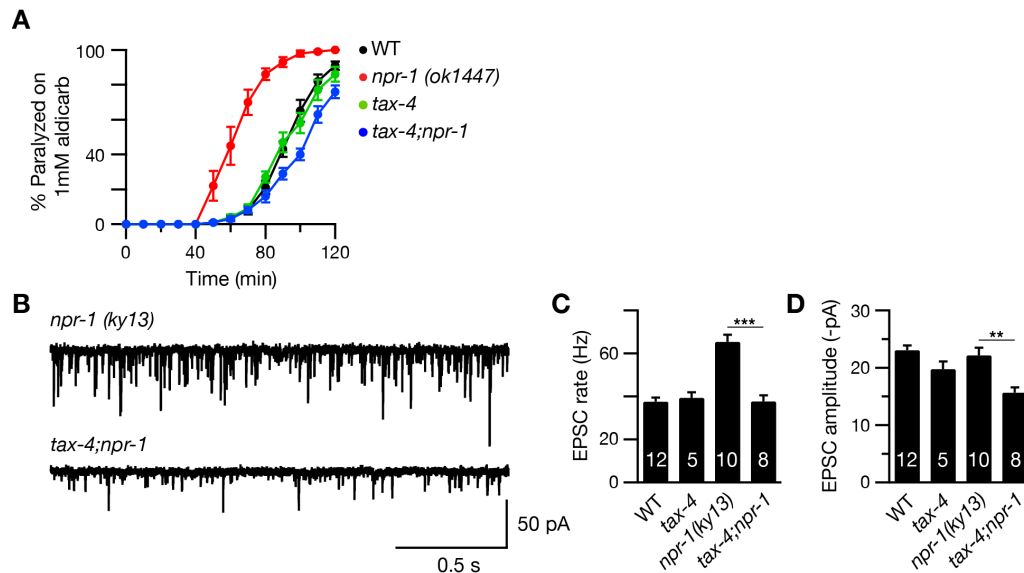


Figure 3.4. Sensory transduction mediated by TAX-4/CNG channels is required for sensory-evoked enhancement of cholinergic transmission in *npr-1* mutants.

(A) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=7 trials for all the genotypes, and 20-30 worms per trial; wild-type, *npr-1(ok1447)*, *tax-4(p678)*, and *tax-4;npr-1*). The *npr-1* aldicarb hypersensitivity was blocked by mutations inactivating TAX-4/CNG channels. (B-D) Endogenous EPSCs were recorded from body wall muscles of the adult worms for the indicated genotypes. Representative traces of endogenous EPSCs (B) and summary data are shown (C, D). The *npr-1* cholinergic transmission defect was abolished by mutations inactivating TAX-4. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$; **, $p < 0.01$).

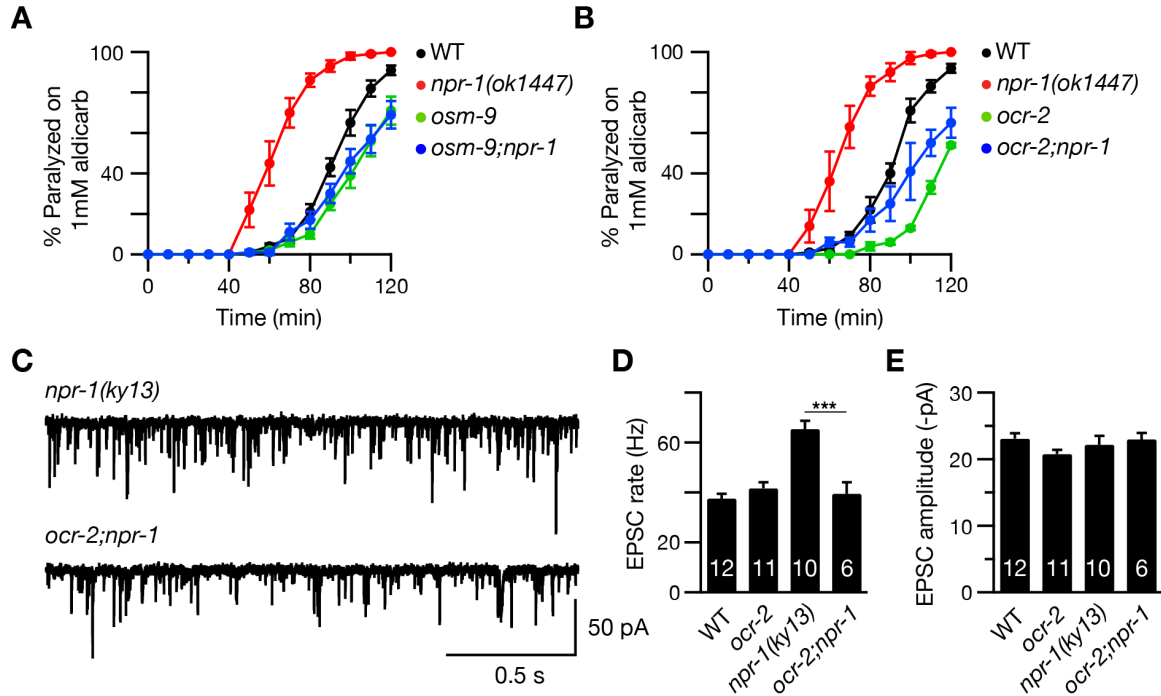


Figure 3.5. Sensory transduction mediated by OSM-9 and OCR-2/TRPV channels is required for sensory-evoked enhancement of cholinergic transmission in *npr-1* mutants.

(A-B) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotype (n=7, 4, 7, 4, 6, 3, 6, and 3 trials for wild-type (A), wild-type (B), *npr-1(ok1447)* (A), *npr-1(ok1447)* (B), *osm-9(ky10)*, *ocr-2(ak47)*, *osm-9;npr-1*, and *ocr-2;npr-1* animals, respectively, and 20-30 worms per trial). The *npr-1* aldicarb hypersensitivity was blocked by mutations inactivating OSM-9 and OCR-2/TRPV channels. (C-E) Endogenous EPSCs were recorded from body wall muscles of the adult worms for the indicated genotypes. Representative traces of endogenous EPSCs (C) and summary data are shown (D, E). The *npr-1* cholinergic transmission defect was abolished by mutations inactivating OCR-2. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

mutants was caused by heightened sensory activity in the RMG circuit.

Glutamate signaling mediates sensory-evoked enhancement of cholinergic transmission at NMJs

How can heightened sensory activity be transmitted to body wall muscles and enhance cholinergic transmission at NMJs in *npr-1* mutants? Glutamate and neuropeptides are two major types of neurotransmitters expressed in sensory neurons in the RMG circuit. In *C. elegans*, glutamate transmission is mostly utilized at synapses between sensory neurons and downstream interneurons including command interneurons that further make synapses onto motor neurons and modulate locomotion of animals (Brockie and Maricq, 2006). Glutamate-mediated fast synaptic transmission at sensory-interneuron synapses is required for behavioral responses to noxious stimuli such as nose touch, hyperosmotic stress, and chemical repellents (Hart et al., 1999; Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002). To determine if glutamate transmission mediates the enhancement of cholinergic transmission in body muscles of *npr-1* mutants, we examined mutants lacking *eat-4* vGLUT (vesicular Glutamate Transporter) in which glutamate transmission is disrupted. Inactivating EAT-4 suppressed aldicarb hypersensitivity and abolished cholinergic transmission defect of *npr-1* mutants (Fig. 3.6), indicating that glutamate transmission is indeed required for sensory-evoked enhancement of cholinergic transmission at NMJs.

Among sensory neurons in the RMG circuit, ASH and ASK neurons release glutamate as well as neuropeptides for transmission to downstream interneurons, as evidenced by expression of EAT-4/vGLUT in these two neurons (Lee et al., 1999). Two

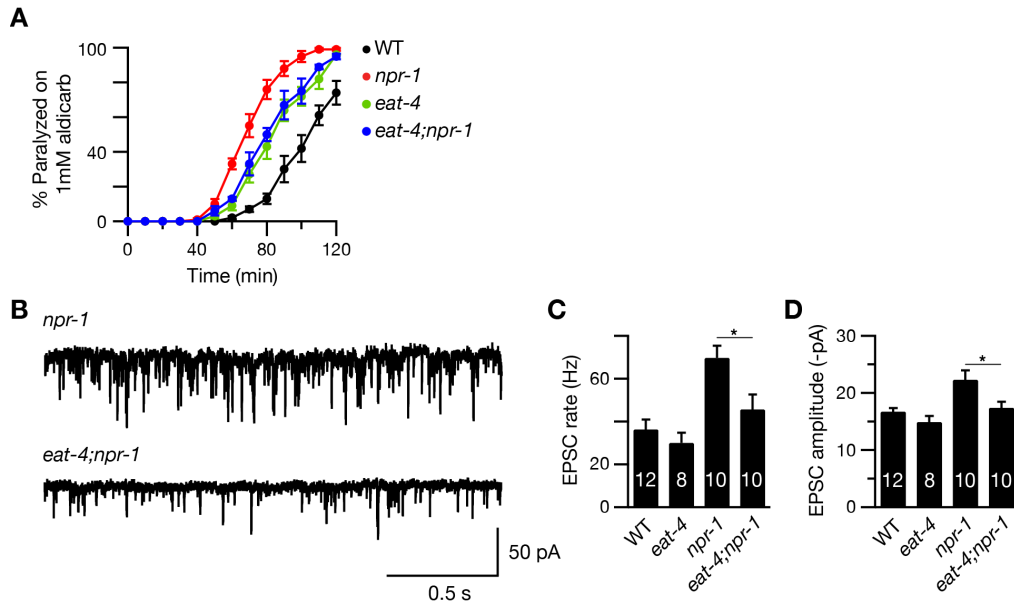


Figure 3.6. Glutamate transmission mediates sensory-evoked enhancement of cholinergic transmission in *npr-1* mutants.

(A) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes ($n=5$ trials for all the genotypes, and 20-30 worms per trial; wild-type, *npr-1*(*ky13*), *eat-4*(*ky5*), and *eat-4;npr-1*). The *npr-1* aldicarb hypersensitivity was suppressed by mutations inactivating EAT-4/vGLUT. (B-D) Endogenous EPSCs were recorded from body wall muscles of the adult worms for the indicated genotypes. Representative traces of endogenous EPSCs (B) and summary data are shown (C, D). The *npr-1* cholinergic transmission defect was abolished by mutations inactivating EAT-4. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$).

lines of evidence support the idea that glutamate release from ASH and ASK mediates sensory-evoked enhancement of cholinergic transmission at NMJs. First, *eat-4* transgene expressed in either ASH or ASK sensory neurons (using the *sra-6* promoter and the *sra-9*

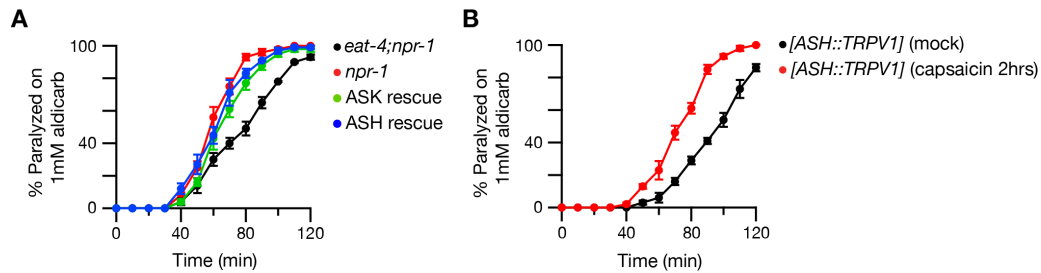


Figure 3.7. Glutamate release from ASH and ASK sensory neurons mediates sensory-evoked enhancement of cholinergic transmission in *npr-1* mutants.

(A-B) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=4, 3, 4, 4, 5, and 5 trials for *npr-1(ky13)*, *eat-4(ky5);npr-1*, ASH rescue, ASK rescue, *[ASH::TRPV1]* (mock), and *[ASH::TRPV1]* (capsaicin 2hrs), respectively, and 20-30 worms per trial). (A) The *npr-1* aldicarb hypersensitivity was reinstated by transgenes expressing EAT-4 in ASH (*sra-6* promoter) and ASK (*sra-9* promoter), using the indicated promoters. (B) Forced depolarization of ASH increased aldicarb sensitivity. Time course of paralysis of transgenic worms expressing rat TRPV1 channels in ASH were analyzed with or without capsaicin treatment (2 hours).

promoter, respectively) reinstated aldicarb hypersensitivity in *eat-4;npr-1* double mutants (Fig. 3.7A). Second, chronic activation of ASH sensory neurons induced aldicarb hypersensitivity. A prior study showed that capsaicin treatment depolarizes ASH neurons expressing rat TRPV1 channels and induces backing responses in these transgenic animals (Tobin et al., 2002). Prolonged treatment of capsaicin induced aldicarb hypersensitivity in these transgenic animals (Fig. 3.7B), suggesting that forced depolarization of ASH sensory neurons was sufficient to enhance cholinergic transmission at NMJs. Electrophysiological recordings on capsaicin treated animals should further confirm this idea.

GLR-2 is the major glutamate receptor that mediates sensory-evoked enhancement of cholinergic transmission at NMJs

Similar to glutamate transmission in vertebrates, glutamate-gated current in *C. elegans* consists of two components with different kinetic properties. A large and rapid component is dependent on GLR-1 and GLR-2/AMPA-type glutamate receptors, while a small and long-lasting component is dependent on NMR-1/NMDA-type glutamate receptors (Mellem et al., 2002). Mutants lacking each type of glutamate receptor show differences in sensory behaviors, suggesting that glutamate receptor composition and function can dictate distinct behavioral outcomes. For example, mutants lacking either GLR-1 or GLR-2 have defects in both nose touch responses and osmotic avoidance, whereas mutants lacking NMR-1 has a subtle defect only in osmotic avoidance (Mellem et al., 2002). In order to identify which glutamate receptors play a major role in mediating sensory-evoked enhancement of cholinergic transmission at NMJs, we first examined aldicarb sensitivity of *glr-1*, *glr-2*, and *nmr-1* single mutants as well as each double mutant with *npr-1* mutations. Two different mutations inactivating GLR-2 consistently blocked aldicarb hypersensitivity in *npr-1* mutants, whereas mutations inactivating GLR-1 or NMR-1 had a subtle or no effect, respectively (Fig. 3.8). These results suggest that GLR-2 is a major glutamate receptor that mediates sensory-evoked enhancement of cholinergic transmission at NMJs, although electrophysiological recordings should confirm these results.

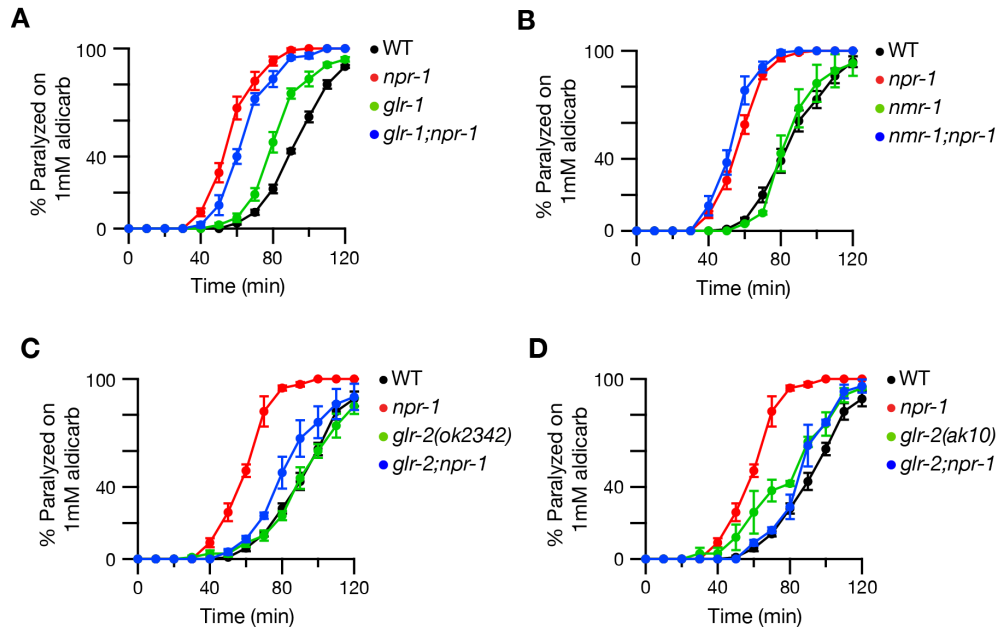


Figure 3.8. GLR-2 is the major glutamate receptor that mediates sensory-evoked enhancement of cholinergic transmission in *npr-1* mutants.

(A-D) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=5, 6, 4, 4, 7, 3, 4, 3, 4, 2, 4, 3, 4, and 2 trials for wild-type (A), wild-type (B), wild-type (C, D), *npr-1(ky13)* (A), *npr-1(ky13)* (B), *npr-1(ky13)* (C, D), *glr-1(n2461)*, *nmr-1(ak4)*, *glr-2(ok2342)*, *glr-2(ak10)*, *glr-1;npr-1*, *nmr-1;npr-1*, *glr-2(ok2342);npr-1*, *glr-2(ak10);npr-1* animals, respectively, and 20-30 worms per trial). (A, B) The *npr-1* aldicarb hypersensitivity was minimally and not affected by mutations inactivating GLR-1 (A) and NMR-1 (B), respectively. (C, D) The *npr-1* aldicarb hypersensitivity was greatly suppressed by two different mutations inactivating GLR-2.

Neuropeptides also mediate sensory-evoked increase in cholinergic transmission

Neuropeptides modulate neuronal activity, animal behaviors, and diverse physiological processes in both vertebrate and invertebrate nervous system (Taghert and Nitabach, 2012; van den Pol, 2012). *C. elegans* expresses over 110 neuropeptide genes encoding

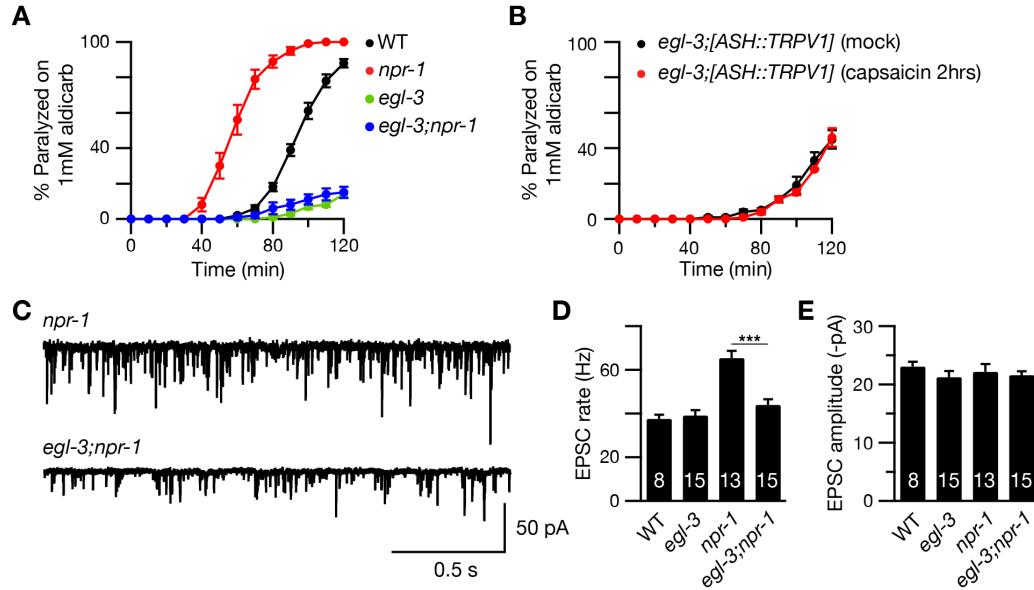


Figure 3.9. Neuropeptides mediate sensory-evoked enhancement of cholinergic transmission in *npr-1* mutants.

(A-B) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=12, 12, 6, 6, 4, and 4 trials for wild-type, *npr-1*(*ky13*), *egl-3*(*nr2090*), *egl-3;npr-1*, *egl-3*;[ASH::TRPV1] (mock), and *egl-3*;[ASH::TRPV1] (capsaicin 2hrs), respectively, and 20-30 worms per trial). (A) The *npr-1* aldicarb hypersensitivity was blocked by mutations inactivating EGL-3/PC2. (B) Forced depolarization of ASH had no effect on aldicarb sensitivity in *egl-3* mutants. Time course of paralysis of transgenic worms were analyzed with or without capsaicin treatment (2 hours). (C-E) Endogenous EPSCs were recorded from body wall muscles of the adult worms for the indicated genotypes. Representative traces of endogenous EPSCs (C) and summary data are shown (D, E). The *npr-1* cholinergic transmission defect was abolished by mutations inactivating EGL-3. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

over 250 distinct neuropeptides (Li and Kim, 2008), and their role in modulating neural circuits and behaviors such as locomotion, feeding, and reproduction have been recently studied at the circuit level (Barrios et al., 2012; Beets et al., 2012; Chalasani et al., 2010; Garrison et al., 2012; Hu et al., 2011b).

Most of sensory neurons and interneurons, including neurons in the RMG circuit, release one or more neuropeptides. Thus, it is possible that heightened sensory activity in *npr-1* mutants might increase the secretion of neuropeptides from the RMG circuit that modulate neurotransmitter release at cholinergic synapses in body muscles. Consistent with this idea, the *npr-1* aldicarb hypersensitivity and cholinergic transmission defect were eliminated by mutations inactivating *egl-3* PC2 (Fig. 3.9A and C-E), which is required for pro-neuropeptide processing (Husson et al., 2006; Kass et al., 2001). In addition, forced depolarization of ASH failed to enhance aldicarb sensitivity in *egl-3* mutants (Fig. 3.9B). These results suggest that the *npr-1* cholinergic transmission defect was mediated by an endogenous neuropeptide.

Sensory-evoked enhancement of cholinergic transmission is partially mediated by PDF-1 and PDFR-1

We first hypothesized that PDF-1 secreted from the RMG circuit might enhance cholinergic transmission at NMJs in *npr-1* mutants for two reasons. First, PDF-1 secretion was increased in *npr-1* adult animals (Fig. 2.15). Second, in addition to touch neurons and body wall muscles, *pdf-1* promoter is also expressed in AVD and PVC command interneurons that make synapses onto cholinergic motor neurons (Barrios et al., 2012). Consistent with this idea, both mutations inactivating PDF-1 and PDFR-1

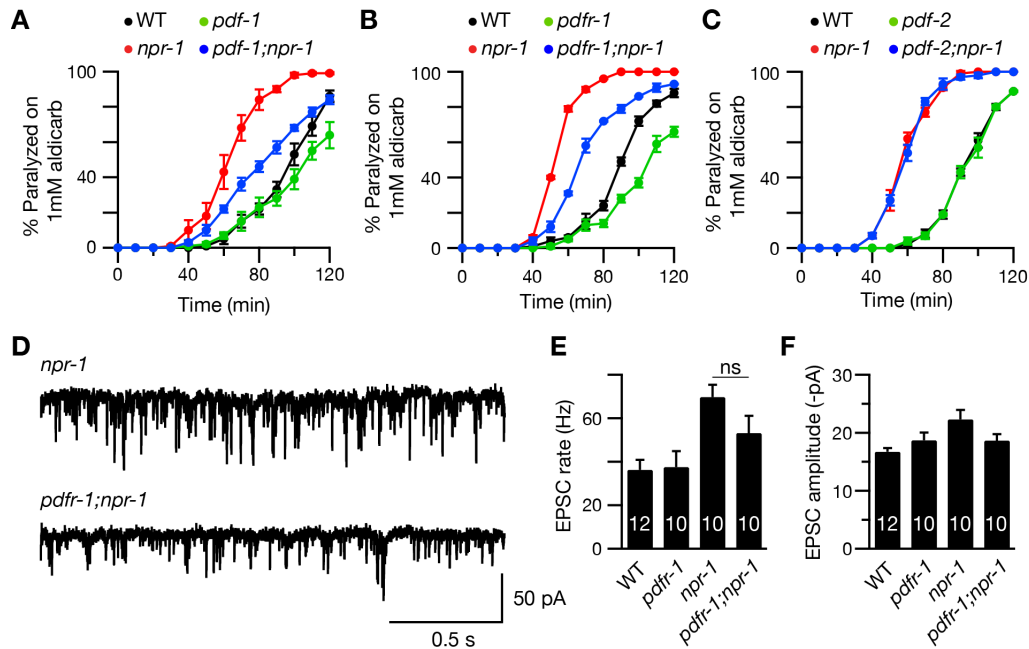


Figure 3.10. PDF-1 and PDFR-1 partially mediate sensory-evoked enhancement of cholinergic transmission in *npr-1* mutants.

(A-C) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=6, 3, 6, 3, 6, 3, 3, 6, 3, and 3 trials for wild-type (A), wild-type (B, C), *npr-1*(*ky13*) (A), *npr-1*(*ky13*) (B, C), *pdf-1*(*tm1996*), *pdf-1*(*ok3425*), *pdf-2*(*tm4393*), *pdf-1;npr-1*, *pdfr-1;npr-1*, *pdf-2 npr-1* animals, respectively, and 20-30 worms per trial). The *npr-1* aldicarb hypersensitivity was partially suppressed by mutations inactivating PDF-1 (A) and PDFR-1 (B), but not by those inactivating PDF-2 (C). (D-F) Endogenous EPSCs were recorded from body wall muscles of the adult worms for the indicated genotypes. Representative traces of endogenous EPSCs (D) and summary data are shown (E, F). The *npr-1* cholinergic transmission defect was partially suppressed by mutations inactivating PDFR-1, although more animals should be analyzed to test statistical significance. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (ns, not significant).

partially suppressed aldicarb hypersensitivity in *npr-1* mutants, whereas inactivating PDF-2 had no effect on the *npr-1* defect (Fig. 3.10A-C). Furthermore, mutations inactivating PDFR-1 also partially suppressed the *npr-1* cholinergic transmission defect (Fig. 3.10D-F). Although more animals should be analyzed to test statistical significance, these results suggest a possibility that sensory-evoked enhancement of cholinergic transmission is partially mediated by PDF-1.

Identification of potential neuropeptides that mediate sensory-evoked enhancement of cholinergic transmission

In *C. elegans*, mature neuropeptides are produced by a series of posttranslational modifications of precursor peptides, which involve activation of EGL-3/PC2 by SBT-1/Chaperonin, cleavages of precursors by EGL-3/PC2, and removal of basic residues by EGL-21/Carboxypeptidase (Jacob and Kaplan, 2003; Kass et al., 2001; Lindberg et al., 1998). In addition to EGL-3, mutations inactivating EGL-21 and SBT-1 also suppressed aldicarb hypersensitivity of *npr-1* mutants (Fig. 3.11). However, inactivating SBT-1 was much less effective in blocking the *npr-1* defect than inactivating EGL-3 or EGL-21 (Fig. 3.9A and 3.11). According to mass spectrometry studies on neuropeptide expression profile in *egl-3*, *egl-21*, and *sbt-1* mutants, neuropeptides encoded by 16 *nlp* and *flp* neuropeptide genes were still present in *sbt-1* mutants, but not in *egl-3* and *egl-21* mutants (Husson et al., 2006; Husson et al., 2007; Husson and Schoofs, 2007). Based on these results, we reasoned that one or more of neuropeptides encoded by these 16 genes might mediate sensory-evoked enhancement of cholinergic transmission. Thus, we performed a small-scale RNAi screen for neuropeptide genes whose knockdown causes

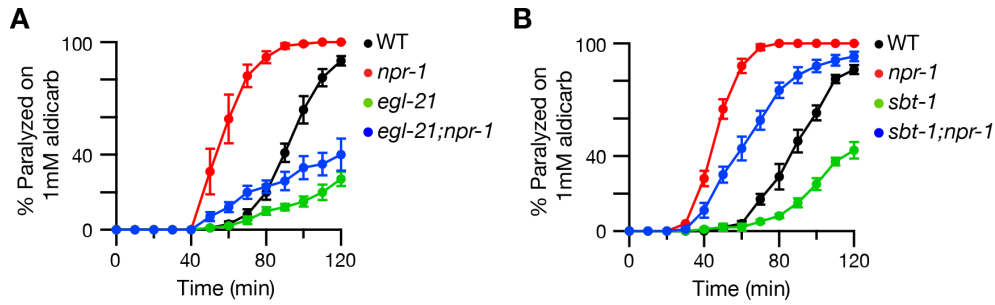


Figure 3.11. SBT-1 minimally affects the *npr-1* aldicarb hypersensitivity.

(A-B) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=6, 6, 5, 6, 4, 6, and 5 trials for wild-type, *npr-1(ky13)* (A), *npr-1(ky13)* (B), *egl-21(n476)*, *sbt-1(ok901)*, *egl-21;npr-1*, and *sbt-1;npr-1* animals, respectively, and 20-30 worms per trial). Mutations inactivating SBT-1 (A) had much less effect on the *npr-1* aldicarb hypersensitivity than inactivating EGL-21 (B).

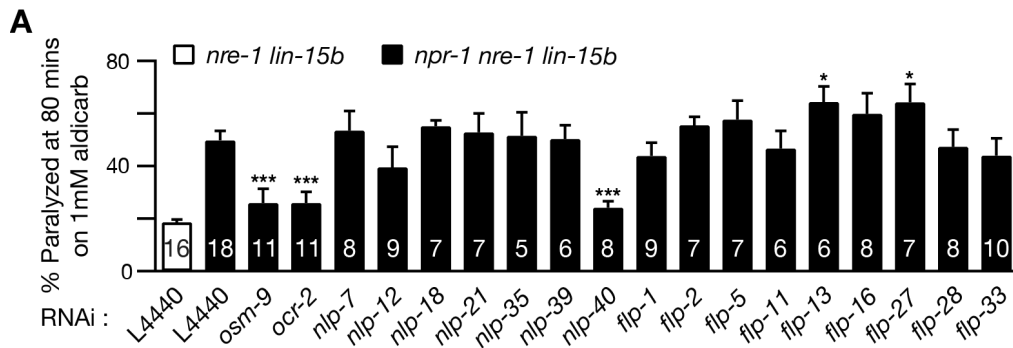


Figure 3.12. RNAi screen for neuropeptide genes whose knockdown suppresses the *npr-1* aldicarb hypersensitivity.

(A) Percentages of worms paralyzed at 80 minutes on 1 mM aldicarb were plotted following treatment with the indicated RNAi clones. RNAi was carried out using RNAi hypersensitive strains (*nre-1 lin-15b*). Knockdown of *osm-9* and *ocr-2* was served as positive control. Knockdown of *nlp-40* significantly suppressed the *npr-1* aldicarb hypersensitivity, whereas knockdown of 15 other neuropeptides had little effect the *npr-1* defect. L4440 indicates the empty vector control. The number of trials is indicated for each RNAi clone. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; ***, $p < 0.001$).

suppression of aldicarb hypersensitivity of *npr-1* mutants. Only one, *nlp-40*, out of 16 neuropeptide genes showed whose knockdown significantly suppressed aldicarb hypersensitivity of *npr-1* mutants (Fig. 3.12). To validate the RNAi screen results, we examined aldicarb sensitivity of *nlp-40;npr-1* double mutants as well as *nlp-12;npr-1* and *flp-1;npr-1*, which showed slight suppression of *npr-1* aldicarb hypersensitivity in the RNAi screen. All showed only small suppression of *npr-1* aldicarb hypersensitivity (Fig. 3.13), suggesting that these neuropeptides either partially mediate or have no effect on the *npr-1* cholinergic transmission. However, further electrophysiological analyses should lead to more solid conclusion.

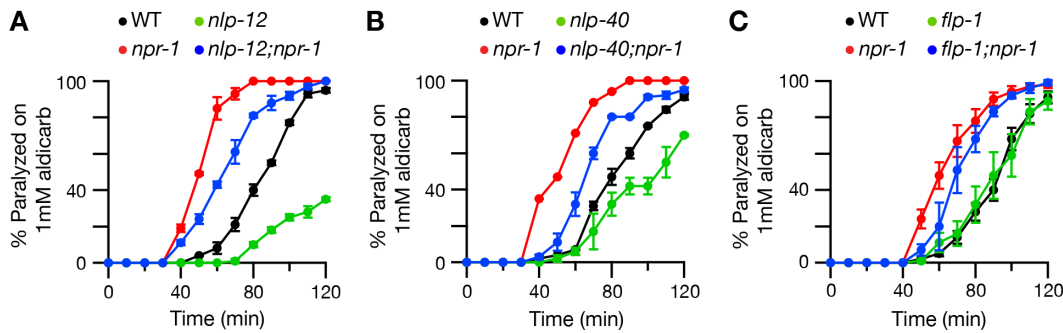


Figure 3.13. Potential neuropeptide genes that might mediate sensory-evoked enhancement of cholinergic transmission in *npr-1* mutants.

(A-C) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=3, 2, 7, 2, 1, 4, 3, 2, 4, 3, 3, and 4 trials for wild-type (A), wild-type (B), wild-type (C), *npr-1(ky13)* (A), *npr-1(ky13)* (B), *npr-1(ok1447)* (C), *nlp-12(ok335)*, *nlp-40(tm4085)*, *flp-1(yn4)*, *nlp-12;npr-1*, *nlp-40;npr-1*, and *flp-1;npr-1* animals, respectively, and 20-30 worms per trial). Each mutation inactivating NLP-12 (A), NLP-40 (B), and FLP-1 (C) had a small suppression of the *npr-1* aldicarb hypersensitivity.

DISCUSSION

We describe a circuit mechanism controlling sensory-evoked enhancement of cholinergic transmission at *C. elegans* NMJs. Heightened RMG circuit activity in *npr-1* mutants increases endogenous cholinergic transmission in body muscles. Both glutamate and neuropeptide mediate the sensory-evoked enhancement of cholinergic transmission. Glutamate released from the RMG circuit neurons (ASH and ASK) activates GLR-2 in the postsynaptic neurons, and eventually increases cholinergic transmission at NMJs. Below we discuss the significance and future directions of these results.

Our results suggest that heightened sensory activity in the RMG circuit increases glutamate release from ASH and ASK sensory neurons. What postsynaptic neurons are target of glutamate released from these neurons? Inactivating GLR-2 had a much greater effect on suppression of the *npr-1* aldicarb hypersensitivity than inactivating GLR-1 (Fig 3.8). Thus, neurons in which GLR-2, not GLR-1, carries most of the glutamate-gated currents are potential targets. Expression analyses on ionotropic glutamate receptors in *C. elegans* revealed that 3 neurons (AIA, RIA, and DVA) express GLR-2, but not GLR-1, and that GLR-2 may form homomeric receptors in these neurons (Aronoff et al., 2004; Brockie et al., 2001). Among these neurons, AIA, an amphid interneuron, receives direct synaptic inputs from both ASH and ASK sensory neurons. Thus, GLR-2 may function in AIA to mediate sensory-evoked enhancement of cholinergic transmission at NMJs. PLM touch neurons that mediate touch sensation in the posterior half of worms also release glutamate. Interestingly, DVA, a stretch sensitive sensory neuron, receives direct synaptic inputs from PLM. Considering that PDF-1 enhances touch-evoked calcium transients in

ALM (touch neuron that mediates touch sensation in the anterior half of worms) in *npr-1* mutants (Fig 2.21 and 2.22), touch-evoked calcium transients in PLM may also be increased in *npr-1* mutants through the action of PDF-1 secreted from the RMG circuit. Therefore, in *npr-1* mutants, GLR-2 mediated fast transmission in DVA may also be indirectly increased by sequential action of PDF-1 activation of PLM and glutamate release from PLM. Consistent with this idea, inactivating PDFR-1 partially suppressed the *npr-1* cholinergic transmission defect (Fig 3.11). Together, these results suggest that GLR-2 may mediate sensory-evoked enhancement of cholinergic transmission by functioning in AIA and DVA neurons. This idea should be further addressed by site-of-action experiments, using AIA and DVA specific expression of GLR-2 in *glr-2;npr-1* double mutants.

Inactivating EGL-3/PC2 completely abolished the *npr-1* cholinergic transmission defect (Fig 3.9), suggesting that neuropeptides are required for sensory-evoked enhancement of cholinergic transmission in body muscles. Then what neuropeptides mediate this process? First, PDF-1 may be involved in this process in a way that it increases touch neuron activity as discussed above. Second, given that AIA and DVA are potential recipients of glutamatergic output from the RMG circuit, neuropeptides expressed in these neurons may mediate sensory-evoked enhancement of cholinergic transmission. AIA expresses FLP-1, FLP-2, and INS-1 neuropeptides, while DVA expresses NLP-12. In the previous RNAi screen, both *flp-1* and *nlp-12* were identified whose inactivation caused resistance to aldicarb (Sieburth et al., 2005). (In this study, *flp-1* mutations had no effect on aldicarb sensitivity (Fig 3.13), perhaps because the *flp-1* deletion allele (*yn4*) also inactivates a neighboring gene *daf-10* encoding an intraflagellar

transport complex component). Furthermore, stretch-induced secretion of NLP-12 from DVA neuron increases endogenous EPSC frequency by activating its receptor CKR-2 in cholinergic motor neurons (Hu et al., 2011b). Interestingly, NLP-12 secretion from DVA was increased in *npr-1* mutants (Hu et al., 2011b). These results suggest that FLP-1 from AIA and NLP-12 from DVA may mediate the *npr-1* cholinergic transmission defect. However, inactivating FLP-1 and NLP-12 showed rather a small suppression of the *npr-1* aldicarb hypersensitivity (Fig. 3.13). Thus, electrophysiological recordings should further clarify the idea.

What is the physiological role of sensory-evoked enhancement of cholinergic transmission at NMJs? Excitatory synaptic inputs are provided by cholinergic synapses in body muscles in *C. elegans*. Thus, the strength of cholinergic transmission determines body muscle excitability, thereby altering speed of worms. For example, *goa-1* G_o protein mutants have increased endogenous EPSC rate and concomitant increase in locomotion rate (Segalat et al., 1995; Vashlishan et al., 2008). Conversely, *unc-57* Endophilin mutants have decreased endogenous EPSC rate and concomitant decrease in locomotion rate (Bai et al., 2010). These results provide a potential correlation between the frequency of endogenous cholinergic transmission and speed of worms. Likewise, *npr-1* mutants have increased locomotion rate as adults, which is rescued by transgenes expressing NPR-1 in the RMG circuit, and suppressed by inactivating TAX-4/CNG, OSM-9 and OCR-2/TRPV sensory transduction channels (Coates and de Bono, 2002; de Bono et al., 2002; Macosko et al., 2009). Thus, enhanced cholinergic transmission may contribute to the increase in locomotion rate in *npr-1* mutants.

NPR-1 inhibits activity of sensory neurons in the RMG circuit that mediate responses to repellent cues such as hyperoxia, pheromone, and aversive odor and chemicals. Fast synaptic transmission carried by glutamate mediates acute avoidance responses to aversive stimuli (Hart et al., 1999; Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002). By contrast, our results suggest that concerted action of glutamate and neuropeptide may cause long-term change in body muscle activity and speed of worms in response to prolonged exposure to noxious environment. Both inactivation of NPR-1 and forced activation of ASH neurons (Fig 3.7) would mimic the prolonged exposure to aversive stimuli.

One of the interesting future experiments is to examine the effect of forced activation of other sensory neurons on synaptic transmission in body muscles. AWC neurons detect attractive odors and mediate chemotactic behavior (Bargmann et al., 1993). The attractive odors that AWC can sense include volatile organic compounds such as benzaldehyde, butanone, and isoamyl alcohol, which correspond to almond, butterscotch, and banana odors, respectively (Bargmann et al., 1993). Preliminary experiments showed that prolonged capsaicin treatment of transgenic animals expressing TRPV1 in AWC neurons decreased aldicarb sensitivity (Fig 3.14). These preliminary results suggest that forced activation of AWC may cause decrease in cholinergic transmission in body muscles, although electrophysiological recordings should further identify the source of the aldicarb resistance. These results also suggest a possibility that body muscle activity and locomotion rate may be decreased in response to prolonged exposure to attractive sensory cues such as food odors.

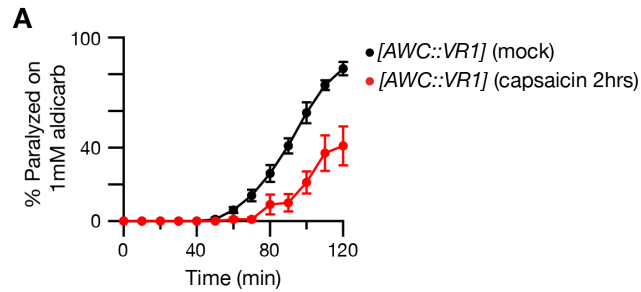


Figure 3.14. Chronic activation of AWC neurons decreases aldicarb sensitivity.

(A) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=4 trials for all the genotypes, and 20-30 worms per trial). Forced depolarization of AWC decreased aldicarb sensitivity. Time course of paralysis of transgenic worms expressing rat TRPV1 channels in AWC were analyzed with or without capsaicin treatment (2 hours).

Together, our results suggest that *C. elegans* may adapt to new environment by changing synaptic activity in body muscles and altering locomotion rate. Increased body muscle activity and locomotion would be beneficial for escaping from noxious environment. Conversely, decreased body muscle activity and locomotion would be beneficial for staying in attractive environment. This study will provide insight on circuit mechanism underlying sensory-evoked long-term synaptic plasticity and corresponding behavioral changes.

MATERIALS AND METHODS

Strains

Strain maintenance and genetic manipulation were performed as described (Brenner, 1974). Animals were cultivated at 20°C on agar nematode growth media (NGM) seeded with OP50 (for imaging and behavior) or HB101 *E.coli* (for electrophysiology). Wild type reference strain was N2 Bristol. Strains used in this study are as follows:

Mutant strains and integrants

KP6048 *npr-1(ky13)* *X*

DA609 *npr-1(ad609)* *X*

KP6064 *npr-1(ok1447)* *X*

KP6072 *flp-18(tm2179)* *X*

KP6077 *flp-21(pk1601)* *V*

PR678 *tax-4(p678)* *III*

KP3183 *osm-9(ky10)* *IV*

CX4544 *ocr-2(ak47)* *IV*

MT6308 *eat-4(ky5)* *III*

KP0004 *glr-1(n2461)* *III*

VM487 *nmr-1(ak4)* *II*

KP6590 *glr-2(ok2342)* *III*

KP5966 *egl-3(nr2090)* *V*

LSC27 *pdf-1(tm1996)* *III*

KP6340 *pdf-1(ok3425)* *III*

KP6416 *pdf-2(tm4393)* *X*

KP2018 *egl-21(n476) IV*
 KP3387 *sbt-1(ok901) V*
 KP5994 *nlp-12(ok335) I*
 KP6347 *nlp-40(tm4085) I*
 KP6073 *flp-21(pk1601) V; flp-18(tm2179) X*
 KP6067 *flp-18(tm2179) npr-1(ky13) X.*
 KP6071 *flp-21(pk1601) V; npr-1(ky13) X*
 KP6057 *ocr-2(ak47) IV; npr-1(ok1447) X*
 KP6058 *ocr-2(ak47) IV; npr-1(ky13) X*
 KP6059 *osm-9(ky10) IV; npr-1(ok1447) X*
 KP6060 *tax-4(p678) III; npr-1(ky13) X*
 KP6061 *tax-4(p678) III; npr-1(ok1447) X*
 KP6349 *eat-4(ky5) III; npr-1(ky13) X*
 KP6414 *nmr-1(ak4) II; npr-1(ky13) X*
 KP6415 *glr-1(n2461) III; npr-1(ky13) X*
 VM1123 *dpy-19(n1347) glr-2(ak10) III*
 KP6591 *glr-2(ok2342) III; npr-1(ky13) X*
 KP6740 *dpy-19(n1347) glr-2(ak10) III; npr-1(ky13) X*
 KP6054 *egl-3(nr2090) V; npr-1(ky13) X*
 KP6100 *pdf-1(tm1996) III; npr-1(ky13) X*
 KP6410 *pdf-1(ok3425) III; npr-1(ky13) X*
 KP6417 *pdf-2(tm4393) npr-1(ky13) X*
 KP6065 *egl-21(n476) IV; npr-1(ky13) X*

KP6055 *sbt-1(ok901) V;npr-1(ky13) X*

KP5364 *nre-1(hd20) lin-15b(hd126) X*

KP6050 *npr-1(ky13) nre-1(hd20) lin15b(hd126) X*

KP6413 *nlp-12(ok335) I;npr-1(ky13) X*

KP6348 *nlp-40(tm4085) I;npr-1(ky13) X*

KP6076 *daf-10 flp-1(yn4) IV*

KP6082 *daf-10 flp-1(yn4) IV;npr-1(ok1447) X*

CX4978 *kyIS200[sra-6p::VR1, elt-2p::NLS-gfp]* (Gift from Cori Bargmann)

KP6093 *egl-3(nr2090) V;kyIS200[sra-6p::VR1, elt-2p::gfp]*

Strains containing extrachromosomal arrays

CX9396 *npr-1(ad609) X;kyEX1966[flp-21p::npr-1 SL2 GFP, ofm-1p::dsRed]* (Gift from Cori Bargmann)

KP6051 *npr-1(ad609) X;nuEX1519[unc-25p::npr-1::gfp, myo-2p::NLS-mCherry]*

KP6053 *npr-1(ad609) X;nuEX1520[unc-30p::npr-1::gfp, myo-2p::NLS-mCherry]*

KP7149, KP7150 *eat-4(ky5) III; npr-1(ky13) X; nuEX1613-1614[sra-6p::eat-4, myo-2p::NLS-mCherry]*

KP7176, KP7177 *eat-4(ky5) III; npr-1(ky13) X; nuEX1615-1616[sra-9p::eat-4, vha-6p::mCherry]*

KP6430 *nuEX1532[odr-3p::VR1, vha-6p::mCherry]*

Constructs

***eat-4* rescue constructs (*sra-6p::eat-4* (KP#1940) and *sra-9p::eat-4* (KP#1941))**

eat-4 cDNA was amplified by PCR and ligated into expression vectors (pPD49.26) containing the *sra-6* (~3.8kb 5' regulatory sequence: ASH expression) or the *sra-9* (~3kb 5' regulatory sequence: ASK expression) promoters.

Transgenes and germline transformation

Transgenic strains were generated by microinjection of various plasmids with coinjection markers (*myo-2p::NLS-mCherry* (KP#1480) and *vha-6p::mcherry* (KP#1874)). Injection concentration was 40 - 50 ng/μl for all the expression constructs and 10 ng/μl for coinjection markers. The empty vector *pBluescript* was used to bring the final DNA concentration to 100 ng/μl.

Aldicarb assay

Sensitivity to aldicarb was determined by analyzing the time course of paralysis following treatment with 1 mM aldicarb (Sigma-Aldrich) as previously described (Nurrish et al., 1999). Briefly, movement of animals was assessed by prodding animals with a platinum wire every 10 minute following exposure to aldicarb. 20-30 animals were tested for each trial.

RNAi feeding screen

A small-scale RNAi feeding screen was performed as described (Kamath et al., 2003). The screen was performed in the neuronal RNAi hypersensitive mutant background (*nre-1 lin-15b*)(Schmitz et al., 2007). 16 neuropeptide genes whose corresponding neuropeptides are present in *sbt-1* mutants, but absent in *egl-3* and *egl-21* mutants were

selected for the screen (Husson et al., 2006; Husson et al., 2007; Husson and Schoofs, 2007). After 5 days of RNAi treatment (2 generation) at 20°C, 20-30 young adult animals were transferred to 1 mM aldicarb plates, and percentages of worms paralyzed at 80 minutes were scored for each RNAi clone. For comparisons to L4440 empty vector control, statistical significance was determined using two-tailed Student's t test.

Electrophysiology

Electrophysiology was performed on dissected adult worms as previously described (Richmond et al., 1999). Worms were superfused in an extracellular solution containing 127 mM NaCl, 5 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 20 mM glucose, 1 mM CaCl₂, and 4 mM MgCl₂, bubbled with 5% CO₂, 95% O₂ at 20°C. Whole cell recordings were carried out at -60 mV using an internal solution containing 105 mM CsCH₃O₃SC₃, 10 mM CsCl, 15 mM CsF, 4mM MgCl₂, 5mM EGTA, 0.25mM CaCl₂, 10mM HEPES, and 4 mM Na₂ATP, adjusted to pH 7.2 using CsOH. Under these conditions, we only observed endogenous acetylcholine EPSCs. For endogenous GABA IPSC recordings the holding potential was 0 mV, at which we only observe GABAergic postsynaptic currents. All recording conditions were as described (McEwen et al., 2006). Stimulus-evoked EPSCs were stimulated by placing a borosilicate pipette (5–10 µm) near the ventral nerve cord (one muscle distance from the recording pipette) and applying a 0.4 ms, 30 µA square pulse using a stimulus current generator (WPI). Statistical significance was determined using one-way ANOVA with Tukey test for multiple comparison and two-tailed Student's t test for pairwise comparison.

Chapter 4

Concluding Remarks and Future Directions

The experiments discussed in this chapter are unpublished work resulting from collaboration between Seungwon Choi and Zhitao Hu.

Zhitao Hu performed all of the electrophysiological recordings in this chapter, and Seungwon Choi performed all of the other experiments.

IMPLICATIONS OF REGULATING BEHAVIORAL QUIESCENCE AND AROUSAL BY NPR-1

Animals undergo changes in behavioral states such as quiescence and arousal in response to environmental, circadian, or developmental cues. Switch between different behavioral states must be tightly controlled for survival and health of animals. Our study has focused on lethargus behavior, a molting-associated behavioral quiescence in *C. elegans*, and demonstrated that a neuropeptide receptor NPR-1 regulates behavioral quiescence and arousal by modulating the secretion of arousal peptide PDF-1 from central sensory circuit. This study provides new insight on neuropeptide-mediated modulation of sensory-motor circuitry and its role in the precise control of switch between different behavioral states.

Sensory regulation of motor circuit and behavior

Sensory inputs from environment modulate and trigger a variety of behavioral responses in animals. There have been many studies focusing on either the physiology of sensory organs or the motor function of animals. However, it was largely unknown what circuit mechanisms underlie specific sensory-regulated motor behaviors and how and what signaling molecules convey the sensory information to motor circuit.

In this thesis study, we identified two sensory-regulated changes in motor function and behavior, and determined the underlying circuit mechanisms. First, NPR-1 and its ligands, FLP-18 and FLP-21, inhibit PDF-1 secretion from central sensory circuit (the RMG circuit) and dampen peripheral touch neuron activity, thereby promoting

behavioral quiescence during lethargus (Chapter 2). Second, NPR-1 inhibits release of both glutamate and an unknown neuropeptide from central sensory circuit, and reduces cholinergic transmission in body muscles, thereby decreasing locomotion rate in adults (Chapter 3). These results suggest that sensory-evoked neurotransmitter release from central sensory circuit plays a key role in modulating locomotive behavior by adjusting the activity of peripheral motor circuits (touch neurons and body muscles) in *C. elegans*. Our study will contribute to a better understanding of circuit mechanism underlying diverse sensory-regulated motor behavior.

Neuropeptide regulation of neural circuit and behavior

While anatomical connectivity between neurons provides a framework of neural circuitry, unraveling hidden connections between neurons that are mediated by the long-range action of neuromodulators should make the neural circuitry complete. Therefore, study on the neuromodulatory circuitry in the nervous system will lead to a better understanding of circuit function and mechanism for diverse animal physiology and behavior.

Neuromodulators such as neuropeptides, dopamine, and serotonin have a variety of functions in the nervous system from adjusting synaptic strength to altering animal behavior (Marder, 2012). In particular, there is an emerging interest in studying neuropeptide regulation of behavior and underlying circuit mechanism, and many of the recent findings came from studies with genetic model organisms such as flies and worms that are comprised of rather simpler nervous system (Taghert and Nitabach, 2012).

Our study shows that two different neuropeptides play an opposing role in molt-associated behavioral quiescence and arousal. FLP-21 (and FLP-18) promotes behavioral quiescence during molts by activating NPR-1 expressed in the central sensory circuit. Conversely, PDF-1 promotes arousal from behavioral quiescence by activating PDFR-1 expressed in peripheral motor circuits including touch neurons and body muscles. The action of ‘arousal peptide’ PDF-1 is controlled by ‘quiescence peptide’ FLP-21 in that NPR-1 (FLP-21 receptor) inhibits PDF-1 secretion from central sensory circuit, thereby blocking the targeted action of PDF-1 on peripheral motor circuit. These results suggest that switch between behavioral states - quiescence and arousal - is tightly controlled by concerted action of two distinct neuropeptide signaling pathways. Hence, our study will provide new insight on neuropeptide modulation of neural circuits controlling animal physiology and behaviors.

AREAS FOR FUTURE INVESTIGATION

Sensory modalities that promote arousal from lethargus

Our results show that heightened sensory activity in the RMG circuit causes abnormally aroused locomotion during lethargus in *npr-1* mutants. Sensory neurons in the RMG circuit largely mediate responses to environmental repellents such as pheromone, oxygen, and aversive odors and chemicals (Fig 1.1). This sensory gate-keeping function of NPR-1 raises question about which sensory neurons and corresponding sensory modalities play a major role in arousing animals from the molting-associated behavioral quiescence. An approach to address this question is to specifically stimulate each sensory neuron with

known stimulants and see if the stimulation arouses worms from behavioral quiescence. For example, ASK neurons can be stimulated by diverse repertoire of ascarosides (worm pheromone), URX by hyperoxia, ASH by hyperosmolarity or chemical repellents such as copper, and ADL and AWB by aversive odors such as 2-nonanone.

Another approach is to take advantage of inactivating sensory receptor genes that are required for specific sensory perception. Preliminary results suggest a possibility that oxygen can contribute to arousal from lethargus. *gcy-35* encodes a soluble guanylate cyclase that directly binds to molecular oxygen, and regulates foraging behavior by transducing sensory information about ambient oxygen (Cheung et al., 2004; Cheung et al., 2005; Gray et al., 2004). Inactivating GCY-35 blocked the *npr-1* lethargus locomotion defect (Fig 4.1), suggesting that oxygen sensation is required for the *npr-1*

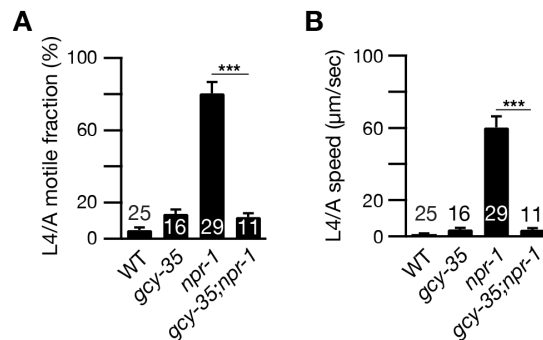


Figure 4.1. Oxygen sensation is required for the *npr-1* lethargus locomotion defect. Locomotion behavior of single worms during the L4/A lethargus was recorded for 30-75 seconds and velocity was measured (2 Hz sampling). Average motile fraction (A), and average locomotion velocity (B) are plotted. The *npr-1* L4/A locomotion quiescence defect was suppressed in double mutants lacking GCY-35/Guanylate cyclase. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

lethargus locomotion defect. Pheromone contribution to arousal from lethargus may be determined by inactivating putative pheromone receptor genes such as *srbc-64*, *srbc-66*, *srg-36*, and *srg-37* (Kim et al., 2009; McGrath et al., 2011).

Regulation of NPR-1 and the ligands

Our results show that NPR-1 and the ligands, FLP-21 and FLP-18, regulate locomotion of worms in and out of lethargus by altering secretion of PDF-1 from the RMG circuit. Then, does lethargus regulate NPR-1 and the ligands, too? If so, expression of NPR-1 and the ligands or secretion of ligands (FLP-18 and FLP-21) should be upregulated during lethargus, when animals are quiescent. We first examined expression level of *npr-1*, *flp-*

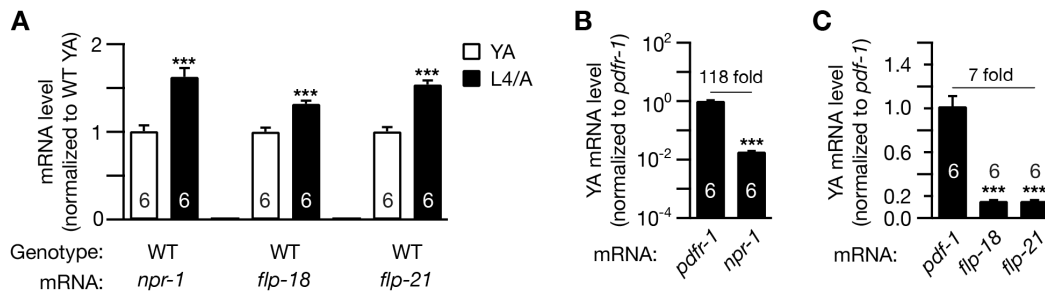


Figure 4.2. The abundance of *npr-1*, *flp-18*, and *flp-21* mRNAs is increased during lethargus.

The abundance of *npr-1*, *flp-18*, and *flp-21* mRNAs in worm extracts was analyzed by quantitative PCR. For each gene, values reported were normalized to those observed in wild type (A), *pdf-1* (B), or *pdf-1* mutant (C) young adults.

(A) The abundance of *npr-1*, *flp-18*, and *flp-21* mRNAs in lethargus was significantly higher than in young adults. (B) The abundance of *npr-1* mRNAs was much lower than that of *pdf-1* mRNAs. (C) The abundance of *flp-18* and *flp-21* mRNAs was much lower than that of *pdf-1* mRNAs. 6 biological replicates were analyzed for each genotype and mRNA. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$; ns, not significant).

21, and *flp-18* in and out of lethargus. The abundance of *npr-1*, *flp-21*, and *flp-18* mRNAs (assayed by quantitative PCR) was increased (~1.5 fold) during the L4/A lethargus (Fig 4.2A). It is hard to believe that ~50% change in the expression of *npr-1* and the ligands exert the huge difference in locomotion in and out of lethargus. However, the basal expression level (in young adults) of *npr-1* mRNA was much lower (~118 fold) than that of *pdf-1* mRNA (Fig 4.2B). Similarly, expression level of *flp-21*, and *flp-18* mRNAs was much lower (~7 fold) than *pdf-1* mRNA (Fig 4.2C). Thus, ~50% change in the expression of low-abundance genes may exert rather big effect on physiology and behavior of animals. Interestingly, expression of *Drosophila* NPF, a NPY homolog in flies, correlates with locomotion state of flies. NPF expression is greatly decreased when fly larvae switch their behavior from continuous feeding (low mobility) to wandering (high mobility) (Wu et al., 2003). Inactivation of NPF signaling increased wandering and decreased feeding, whereas overexpression of NPF extended feeding phase and delayed wandering (Wu et al., 2003). These results suggest that change in NPF expression causes corresponding switch between behavioral states. Nonetheless, the secretion of NPR-1 ligands, FLP-21 and FLP-18, during lethargus should also be further examined.

Downstream effector of PDFR-1 that regulates arousal from behavioral quiescence

Our results show that PDFR-1 functions in both touch neurons and body wall muscles to regulate locomotion of animals. Particularly, in touch neurons, PDFR-1 regulates touch-evoked calcium transients. What molecular mechanism underlies PDFR-1 regulation of touch sensitivity? Because PDFR-1 is coupled to G_s (Janssen et al., 2008), G_s pathway including cAMP and PKA may mediate PDFR-1 regulation of touch sensitivity.

Consistent with this idea, mutants that have high level of cAMP such as *pde-4* Phosphodiesterase and *acy-1(gf)* Adenylate cyclase (gain-of-function) exhibited higher locomotion during the L4/A lethargus than wild type animals (Fig 4.3), although the extent was not as severe as *npr-1* mutants. Consistently, these mutants exhibited increased sensory responsiveness to a repellent odor, 1-octanol, during lethargus in the previous study (Raizen et al., 2008). Furthermore, reduction-of-function mutation of *acy-1* partially suppressed the *npr-1* lethargus locomotion defect, while gain-of-function mutation did not worsen the *npr-1* defect (Fig 4.3). These results suggest that cAMP is a

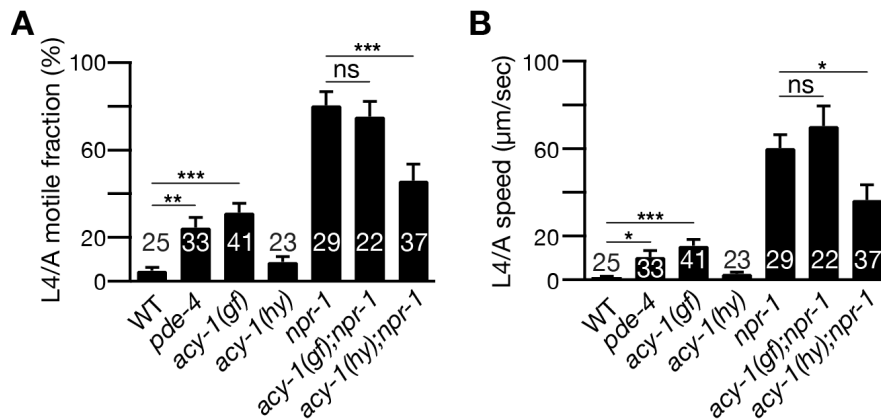


Figure 4.3. cAMP signaling mediates the *npr-1* lethargus locomotion defect.

Locomotion behavior of single worms during the L4/A lethargus was recorded for 30-75 seconds and velocity was measured (2 Hz sampling). Average motile fraction (A), and average locomotion velocity (B) are plotted. *pde-4* (Phosphodiesterase) and *acy-1gf* (Adenylate cyclase) mutants showed increased locomotion during the L4/A lethargus. The *npr-1* L4/A locomotion quiescence defect was partially suppressed by reduction-of-function mutation of *acy-1*, while not altered by gain-of-function mutation of *acy-1*. *gf*, gain-of-function; *hy*, hypomorph. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant).

downstream effector of the *npr-1* lethargus locomotion defect. Site-of-action of PDE-4 and ACY-1 should be further addressed to see if cAMP pathway is indeed the downstream of PDFR-1 in touch neurons and/or body wall muscles.

L-type calcium channels mediate calcium currents in cardiac muscles in a PKA-dependent manner (Kamp and Hell, 2000). Interestingly, touch-evoked calcium responses in ALM touch neurons rely on EGL-19/L-type voltage gated calcium channels (VGCCs) (Suzuki et al., 2003). Therefore, PDFR-1 may regulate touch-evoked calcium transients through PKA and VGCCs.

Synaptic basis of sleep and wakefulness

Synaptic structure and activity are changed during sleep/wakefulness cycles, as demonstrated by biochemistry, imaging and electrophysiological recordings in fly and mammalian brain (Bushey et al., 2011; Gilestro et al., 2009; Nitz et al., 2002; Vyazovskiy et al., 2008). Considering that synaptic activity at NMJs affects locomotion of worms, it will be an interesting question to ask if synaptic activity at NMJs changes between quiescent and aroused state in *C. elegans*. Since it is technically difficult to dissect worms for electrophysiological recordings during the L4/A lethargus, we decided to investigate on other forms of behavioral quiescence in *C. elegans*. There have been two more forms of behavioral quiescence studied in adult stage of worms. First, after prolonged exposure to high-quality food, worms get quiescent in both feeding and movement (called satiety-induced behavioral quiescence), although the quiescence is not as strong as in lethargus (You et al., 2008). Second, overexpression of LIN-3 (epidermal growth factor) induces behavioral quiescence - cessation of feeding and movement - in adult worms, which is as

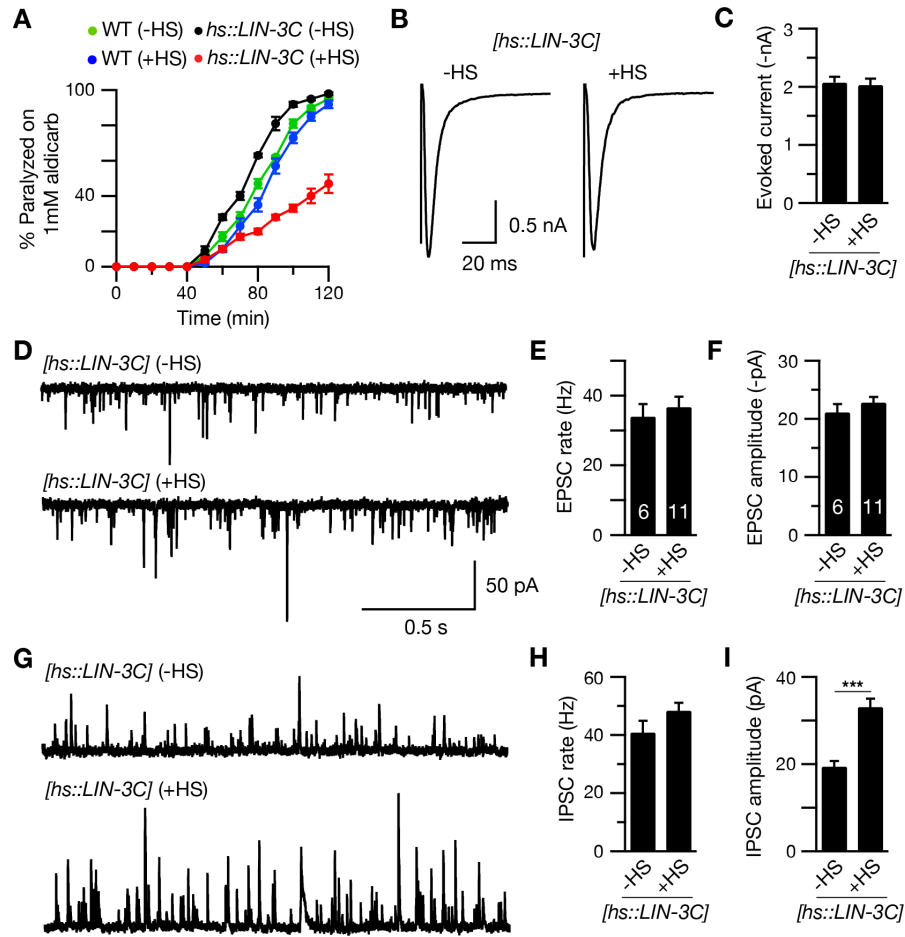


Figure 4.4. LIN-3 overexpression increases endogenous GABA transmission at NMJs.

(A) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=6 trials for all the genotypes, and 20-30 worms per trial). Heat shock-induced overexpression of LIN-3 decreased aldicarb sensitivity. (B-I) Stimulus-evoked EPSCs (B, C), endogenous EPSCs (D-F), and endogenous IPSCs (G-I) were recorded from body wall muscles of the adult worms for the indicated genotypes. Averaged traces of stimulus-evoked EPSCs (B), representative traces of endogenous EPSCs (D) and IPSCs (G), and summary data are shown (C, E, F, H, and I). The amplitude of endogenous EPSCs were increased in *npr-1* mutants (D-F), whereas stimulus-evoked EPSCs and endogenous IPSCs were normal (B, C, G, H, and I). The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (***, $p < 0.001$).

strong as in lethargus (Van Buskirk and Sternberg, 2007). We tested if the latter form of quiescence involves changes in synaptic activity in body muscles. Heat shock promoter-driven overexpression of LIN-3 increased amplitude of endogenous IPSCs, whereas it had no effect on endogenous and stimulus-evoked EPSCs (Fig 4.4). Since endogenous IPSC rate was not significantly altered, LIN-3 overexpression may specifically affect postsynaptic GABA_A receptors that mediate inhibitory currents in body wall muscles in *C. elegans*. Further analyses on LIN-3 overexpression- and satiety-induced behavioral quiescence will provide insight on synaptic basis of behavioral quiescence and arousal.

Pathogen effect on arousal

Numerous studies have shown that sleep and immune function affect each other. Sleep loss interferes with normal immune function, and infection alters sleep pattern (Imeri and Opp, 2009). In particular, it has been shown that infection with various pathogens leads to fragmented NREM (non-rapid eye movement) sleep and reduced REM (rapid eye movement) sleep in mammals including humans (Krueger and Majde, 1994; Krueger et al., 2001). Similarly, in *C. elegans*, *npr-1* mutant animals show increased susceptibility to pathogen *Pseudomonas aeruginosa* strains PA14 (Reddy et al., 2009; Styer et al., 2008) and decreased sleep-like behavior during molts (Chapter 2). These studies suggest that pathogen may disrupt normal quiescence behavior in *C. elegans*. Consistent with this idea, long-term (6-7 hours) exposure to PA14 increased locomotion of worms during the L4/A lethargus (Fig 4.5). PA14 lacking *gacA* (a virulence factor) also significantly increased locomotion during the L4/A lethargus, although the extent to which worms were aroused was slightly lower than PA14 harboring *gacA* (Fig 4.5). These results

suggest that PA14 infection can arouse worms during lethargus, with both virulence factor *gacA*-mediated and non-mediated processes. Interestingly, inactivating PDFR-1 blocked PA14-induced arousal, but not PA14 *gacA* mutant-induced arousal (Fig 4.5), suggesting that PDFR-1 may be specifically required for PA14 *gacA*-mediated arousal during lethargus. Further study on PA14-induced arousal in *C. elegans* will provide insight on interplay between immune and nervous system function.

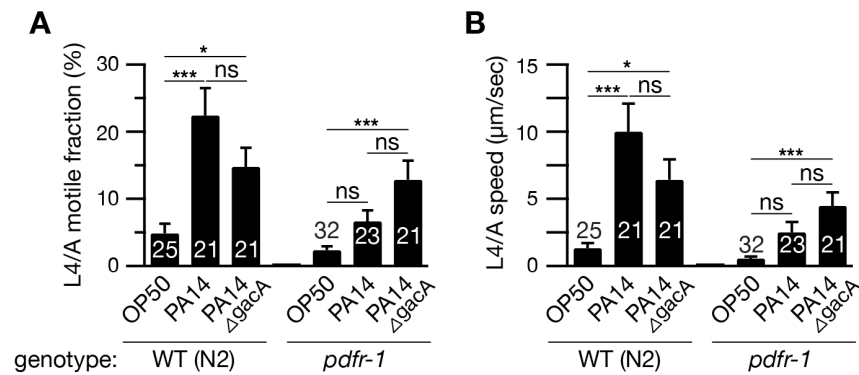


Figure 4.5. PA14 treatment increases locomotion during lethargus.

Locomotion behavior of single worms during the L4/A lethargus was recorded for 30-75 seconds and velocity was measured (2 Hz sampling). Average motile fraction (A), and average locomotion velocity (B) are plotted. Lethargus locomotion was increased by treatment with both PA14 and PA14 lacking *gacA*, a virulence factor, in wild type animals. Mutations inactivating PDFR-1 suppressed hyperlocomotion in PA14 treated worms, but not in worms treated with PA14 lacking *gacA*. The number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated (*, $p < 0.05$; ***, $p < 0.001$; ns, not significant).

Appendix A

Screen (RNAi screen and Mutant screen) for Identification of Neuropeptides

Regulating Lethargus Locomotion Behavior

Seungwon Choi and Kelsey Taylor, a graduate student in Joshua Kaplan lab, performed the RNAi and mutant screens discussed in this appendix when Kelsey was rotating in the lab.

***npr-1* suppressor RNAi screen**

Even though inactivating PDF-1 and PDFR-1 suppressed the *npr-1* lethargus locomotion defect well, the locomotion of *pdf-1;npr-1* and *pdf-1;npr-1* double mutants was slightly higher than *pdf-1* and *pdf-1* single mutants, respectively (Fig 2.10). In contrast, inactivating EGL-3 completely suppressed the *npr-1* lethargus locomotion defect (Fig 2.9). Prompted by these results, we performed RNAi screen for additional neuropeptide genes whose inactivation suppresses the *npr-1* lethargus locomotion defect. We screened 113 neuropeptide genes including 30 *flp* (FMRFamide-related peptides), 39 *ins* (Insulin-like peptides), 43 *nlp* (non-insulin, non-FMRFamide-related peptides) genes, and *pdf-1*, and used *egl-3* RNAi as a positive control. The RNAi screen was performed as described in chapter 2. In the primary screen, 16 genes including *pdf-1* were identified whose inactivation suppressed the *npr-1* lethargus locomotion defect significantly more than did the empty vector control (L4440) ($p < 0.05$, Chi-square test) (Table A.1). To validate the results from the primary screen, we subjected 11 out of the 16 positive genes to the secondary screen. We confirmed that inactivation of 5 neuropeptide genes caused significant suppression of the *npr-1* lethargus locomotion defect compared to empty vector controls (Table A.2). The positive genes include *flp-8*, *nlp-5*, *nlp-10*, *flp-33*, and *pdf-1*. Further analyses on double mutants with *npr-1* mutations should confirm the RNAi screen results.

Neuropeptide mutant screen

Inactivating EGL-3 caused a small, but significant decrease in locomotion quiescence during lethargus (Fig 2.9), suggesting a possibility that there may be a quiescence-

Table A.1

Gene	Wild type-like (%)	npr-1-like (%)	Gene	Wild type-like (%)	npr-1-like (%)	Gene	Wild type-like (%)	npr-1-like (%)
<i>nlp-31</i>	3.45	96.55	<i>ins-1</i>	28.57	71.43	<i>flp-12</i>	40.74	59.26
<i>flp-18</i>	5.88	94.12	<i>ins-17</i>	28.57	71.43	<i>ins-36</i>	40.74	59.26
<i>ins-33</i>	6.67	93.33	<i>ins-19</i>	29.41	70.59	<i>flp-4</i>	40.91	59.09
<i>ins-12</i>	9.09	90.91	<i>flp-3</i>	29.41	70.59	<i>nlp-14</i>	41.38	58.62
<i>flp-19</i>	9.09	90.91	<i>flp-17</i>	30.00	70.00	<i>nlp-15</i>	41.94	58.06
<i>nlp-26</i>	12.00	88.00	<i>flp-21</i>	30.00	70.00	<i>nlp-38</i>	42.50	57.50
<i>ins-37</i>	13.89	86.11	<i>ins-20</i>	30.00	70.00	<i>flp-16</i>	42.86	57.14
<i>ins-29</i>	14.81	85.19	<i>ins-8</i>	30.77	69.23	<i>flp-20</i>	42.86	57.14
<i>nlp-35</i>	15.63	84.38	<i>ins-39</i>	30.77	69.23	<i>nlp-2</i>	42.86	57.14
<i>ins-6</i>	15.79	84.21	<i>nlp-30</i>	30.95	69.05	<i>nlp-6</i>	43.48	56.52
<i>nlp-28</i>	16.67	83.33	<i>nlp-36</i>	31.03	68.97	<i>nlp-7</i>	43.48	56.52
<i>ins-26</i>	16.67	83.33	<i>nlp-24</i>	31.25	68.75	<i>nlp-17</i>	44.00	56.00
<i>flp-11</i>	17.24	82.76	<i>ins-28</i>	31.43	68.57	<i>flp-23</i>	44.44	55.56
<i>ins-27</i>	17.86	82.14	<i>nlp-46</i>	31.58	68.42	<i>ins-5</i>	44.74	55.26
<i>ins-30</i>	17.86	82.14	<i>nlp-29</i>	31.58	68.42	<i>flp7</i>	44.83	55.17
<i>nlp-32</i>	17.95	82.05	<i>nlp-3</i>	32.14	67.86	<i>nlp-9</i>	45.00	55.00
<i>ins-22</i>	18.42	81.58	<i>ins-32</i>	32.14	67.86	<i>ins-16</i>	45.45	54.55
<i>ins-21</i>	18.75	81.25	<i>flp-1</i>	32.26	67.74	<i>flp-28</i>	46.43	53.57
<i>ins-31</i>	19.05	80.95	<i>nlp-39</i>	32.43	67.57	<i>nlp-25</i>	46.67	53.33
<i>flp-26</i>	20.00	80.00	<i>flp-5</i>	33.33	66.67	<i>nlp-11</i>	46.88	53.13
<i>ins-14</i>	20.59	79.41	<i>ins-25</i>	33.33	66.67	<i>nlp-1</i>	47.06	52.94
<i>flp-27</i>	21.05	78.95	<i>flp-15</i>	33.33	66.67	<i>nlp-16</i>	47.06	52.94
<i>ins-18</i>	21.74	78.26	<i>flp-25</i>	33.33	66.67	<i>ins-4</i>	47.22	52.78
<i>flp-24</i>	22.22	77.78	<i>nlp-40</i>	33.33	66.67	<i>flp-6</i>	47.83	52.17
<i>ins-23</i>	22.45	77.55	<i>nlp-33</i>	34.15	65.85	<i>nlp-13</i>	48.28	51.72
<i>daf-28</i>	22.73	77.27	<i>ins-35</i>	34.21	65.79	<i>flp-2</i>	48.65	51.35
<i>ins-3</i>	22.86	77.14	<i>nlp-34</i>	34.48	65.52	<i>flp-8</i>	50.00	50.00
<i>nlp-27</i>	23.68	76.32	<i>nlp-20</i>	35.29	64.71	<i>nlp-10</i>	52.00	48.00
L4440	23.83	76.17	<i>nlp-12</i>	35.48	64.52	<i>ins-9</i>	52.00	48.00
<i>ins-38</i>	24.14	75.86	<i>ins-10</i>	36.36	63.64	<i>flp-13</i>	53.33	46.67
<i>flp-14</i>	25.00	75.00	<i>nlp-21</i>	36.36	63.64	<i>flp-32</i>	54.17	45.83
<i>ins-24</i>	25.00	75.00	<i>ins-34</i>	36.84	63.16	<i>nlp-47</i>	55.17	44.83
<i>ins-13</i>	25.93	74.07	<i>nlp-8</i>	37.50	62.50	<i>pdf-1</i>	57.78	42.22
<i>nlp-23</i>	26.47	73.53	<i>ins-11</i>	37.50	62.50	<i>nlp-4</i>	62.50	37.50
<i>flp-10</i>	26.67	73.33	<i>nlp-22</i>	37.93	62.07	<i>nlp-41</i>	63.89	36.11
<i>flp-22</i>	26.67	73.33	<i>ins-2</i>	37.93	62.07	<i>nlp-18</i>	65.71	34.29
<i>nlp-42</i>	28.21	71.79	<i>flp-9</i>	38.71	61.29	<i>flp-33</i>	70.59	29.41
<i>nlp-19</i>	28.57	71.43	<i>ins-15</i>	38.89	61.11	<i>nlp-5</i>	77.78	22.22
						<i>egl-3</i>	86.56	13.44
L4440: Empty vector control			<i>egl-3</i>: Positive control			Positive hits: $p < 0.05$		

Table A.1. *npr-1* suppressor RNAi screen: Lethargus locomotion behavior (Primary screen). RNAi was carried out using RNAi hypersensitive strains in *npr-1* mutant background (*npr-1 nre-1 lin-15b*). 113 neuropeptide genes were screened. After 2 generation RNAi treatment, worms in lethargus (determined by cessation of pharyngeal pumping) were scored as ‘wild type-like’ (No or little movement) or ‘*npr-1*-like’ (significant movement). 16 genes were identified whose inactivation suppressed the *npr-1* lethargus locomotion defect significantly more than did the empty vector control (L4440) ($p < 0.05$, Chi-square test).

Table A.2

Gene	Wild type-like (%)	<i>npr-1</i> -like (%)	P value
<i>nlp-4</i>	16.67	83.33	0.404516
<i>L4440</i>	29.03	70.97	1
<i>ins-9</i>	41.94	58.06	0.288358
<i>nlp-18</i>	44.44	55.56	0.223135
<i>nlp-41</i>	47.62	52.38	0.172026
<i>flp-32</i>	50.00	50.00	0.093755
<i>nlp-47</i>	53.13	46.88	0.05215
<i>flp-8</i>	60.00	40.00	0.014916
<i>nlp-5</i>	64.71	35.29	0.016502
<i>nlp-10</i>	72.22	27.78	0.003387
<i>egl-3</i>	77.27	22.73	0.000537
<i>flp-33</i>	79.31	20.69	9.58E-05
<i>pdf-1</i>	100.00	0.00	5.15E-05
<i>L4440</i> : Negative control		<i>egl-3</i> : Positive control	
Positive hits: <i>p</i> < 0.05			

Table A.2. *npr-1* suppressor RNAi screen: Lethargus locomotion behavior

(Secondary screen). 11 positive genes from the primary screen were subjected to the secondary screen. Inactivation of 5 neuropeptide genes caused significant suppression of the *npr-1* lethargus locomotion defect compared to empty vector controls (*L4440*) ($p < 0.05$, Chi-square test).

promoting neuropeptide whose inactivation decreases lethargus locomotion quiescence.

Although inactivating either FLP-21 or FLP-18 significantly decreased locomotion quiescence in a Bristol strain expressing low affinity NPR-1(215F) receptors (*npr-1(g320)* mutants) (Fig. 2.5), inactivating FLP-18 and FLP-21, and double mutants inactivating both ligands, had no effect on the L4/A locomotion behavior of worms expressing high affinity NPR-1(215V) receptors (Fig. 2.5). Thus, we carried out an unbiased mutant screen for neuropeptide genes whose inactivation causes decrease in lethargus quiescence. *C. elegans* mutant strains are available for more than half of ~120 identified neuropeptide genes. In the primary screen, 68 neuropeptide mutants were screened, and an only 1 (*flp-1*) out of 68 genes was identified whose inactivation

decreases lethargus quiescence significantly more than did wild type controls ($p < 0.05$, Chi-square test) (Table A.3). However, the effect of *flp-1* mutations on lethargus quiescence was rather subtle compared to *npr-1* mutations. In addition, the deletion allele (*yn4*) that we used to inactivate *flp-1* in the screen also inactivates a neighboring gene *daf-10* (intraflagellar transport complex component). Thus, further analyses on the new allele that specifically inactivate *flp-1* should confirm the screen results. In addition, the effect of the rest of neuropeptides on lethargus behavior should also be further addressed by mutant analyses or RNAi.

Table A.3

Gene	Wild type-like (%)	npr-1-like (%)	Gene	Wild type-like (%)	npr-1-like (%)	Gene	Wild type-like (%)	npr-1-like (%)
<i>Wild type</i>	100.00	0.00	<i>nlp-12</i>	100.00	0.00	<i>flp-6</i>	100.00	0.00
<i>ins-29</i>	100.00	0.00	<i>ins-13</i>	100.00	0.00	<i>flp-7</i>	100.00	0.00
<i>nlp-38</i>	100.00	0.00	<i>flp-27</i>	100.00	0.00	<i>flp-11</i>	100.00	0.00
<i>ins-6</i>	100.00	0.00	<i>flp-14</i>	100.00	0.00	<i>ins-35</i>	100.00	0.00
<i>nlp-18</i>	100.00	0.00	<i>flp-18</i>	100.00	0.00	<i>nlp-3</i>	100.00	0.00
<i>ins-33</i>	100.00	0.00	<i>flp-3</i>	100.00	0.00	<i>flp-28</i>	100.00	0.00
<i>nlp-24</i>	100.00	0.00	<i>nlp-1</i>	100.00	0.00	<i>flp-15</i>	100.00	0.00
<i>ins-7</i>	100.00	0.00	<i>flp-24</i>	100.00	0.00	<i>ins-8</i>	100.00	0.00
<i>nlp-35</i>	100.00	0.00	<i>ins-28</i>	100.00	0.00	<i>ins-31</i>	97.56	2.44
<i>flp-20</i>	100.00	0.00	<i>nlp-7</i>	100.00	0.00	<i>ins-16</i>	97.37	2.63
<i>ins-18</i>	100.00	0.00	<i>ins-14</i>	100.00	0.00	<i>flp-2</i>	97.30	2.70
<i>ins-4</i>	100.00	0.00	<i>ins-27</i>	100.00	0.00	<i>nlp-20</i>	97.22	2.78
<i>ins-19</i>	100.00	0.00	<i>flp-8</i>	100.00	0.00	<i>nlp-36</i>	97.14	2.86
<i>ins-23</i>	100.00	0.00	<i>nlp-23</i>	100.00	0.00	<i>ins-20</i>	96.15	3.85
<i>ins-38</i>	100.00	0.00	<i>flp-25</i>	100.00	0.00	<i>flp-21</i>	96.15	3.85
<i>ins-26</i>	100.00	0.00	<i>flp-5</i>	100.00	0.00	<i>nlp-15</i>	96.15	3.85
<i>ins-22</i>	100.00	0.00	<i>ins-3</i>	100.00	0.00	<i>nlp-29</i>	95.65	4.35
<i>ins-30</i>	100.00	0.00	<i>ins-11</i>	100.00	0.00	<i>nlp-5</i>	93.75	6.25
<i>ins-9</i>	100.00	0.00	<i>flp-33</i>	100.00	0.00	<i>flp-26</i>	93.33	6.67
<i>ins-34</i>	100.00	0.00	<i>flp-16</i>	100.00	0.00	<i>flp-23</i>	92.86	7.14
<i>nlp-9</i>	100.00	0.00	<i>flp-17</i>	100.00	0.00	<i>flp-10</i>	92.59	7.41
<i>nlp-14</i>	100.00	0.00	<i>flp-19</i>	100.00	0.00	<i>daf-28</i>	85.71	14.29
<i>flp-9</i>	100.00	0.00	<i>flp-12</i>	100.00	0.00	<i>flp-1</i>	81.48	18.52
						<i>npr-1</i>	13.48	86.52
<i>Wild type</i> : Negative control			<i>npr-1</i> : Positive control			Positive hits: $p < 0.05$		

Table A.3. Neuropeptide mutant screen: Lethargus locomotion behavior.

68 neuropeptide mutants were screened, and worms in lethargus (determined by cessation of pharyngeal pumping) were scored as ‘wild type-like’ (No or little movement) or ‘*npr-1*-like’ (significant movement). Only 1 (*flp-1*) out of 68 genes was identified whose inactivation decreases lethargus quiescence significantly more than did wild type controls ($p < 0.05$, Chi-square test).

Appendix B

Mutant Screen for Identification of Neuropeptides Altering Aldicarb Sensitivity

Seungwon Choi (Dissertation author) performed all of the experiments discussed in this appendix.

Neuropeptide mutant screen

Inactivating NPR-1 ligands, FLP-18 and FLP-21, and double mutants inactivating both ligands, had no effect on the aldicarb sensitivity in worms expressing high affinity NPR-1(215V) receptors (Fig. 3.2). These results suggest that there may be other endogenous NPR-1 ligands whose inactivation mimics the aldicarb hypersensitivity of *npr-1* mutants, although it is possible that FLP-18 and FLP-21 may function as endogenous ligands in a Bristol strain expressing low affinity NPR-1(215F) receptors (*npr-1(g320)* mutants). Thus, I carried out a mutant screen for neuropeptide genes whose inactivation causes aldicarb hypersensitivity. To identify neuropeptide genes whose inactivation causes aldicarb resistance in the same screen, the aldicarb-induced paralysis of worms was scored at two time points; at 70 minutes for *hic* (hypersensitivity to inhibitors of cholinesterase) genes and at 120 minutes for *ric* (resistance to inhibitors of cholinesterase) genes. In the primary *hic* screen, 27 out of 51 genes (55 alleles) were identified whose inactivation caused significantly higher aldicarb sensitivity than wild type controls ($p < 0.05$, two tailed student t test), and 16 out of the 27 mutants were strong positive hits that exhibited statistically similar extent of aldicarb hypersensitivity to *npr-1* mutants (*hic* controls) ($p > 0.05$ compared to *npr-1* mutants, student t test) (Table B.1). In the primary *ric* screen, 3 (4 alleles) out of 51 genes (55 alleles) were identified whose inactivation caused significantly lower aldicarb sensitivity than wild type controls ($p < 0.05$, two tailed student t test), and 2 out of the 3 mutants were strong positive hits that exhibited statistically similar extent of aldicarb resistance to *egl-3* mutants (*ric* controls) ($p > 0.05$ compared to *egl-3* mutants, student t test) (Table B.2). Most of the screened mutants were collected from C. elegans Genetics Center (CGC) or Mitani lab, and were

Table B.1

% Paralyzed at 70 min	Set 1				
Gene (allele)	Average	SEM	Gene (allele)	Average	SEM
<i>flp-3(ok3265) X.</i>	1.45%	1.18%	<i>flp-15(gk1186) III.</i>	25.44%	8.98%
<i>egl-3 (nr2090) V.</i>	3.17%	1.27%	<i>flp-16(ok3085) II.</i>	28.92%	5.47%
<i>ins-3(ok2478) II.</i>	3.63%	1.63%	<i>ins-17(tm1790) III.</i>	31.83%	1.47%
<i>nlp-18(ok1557) II.</i>	3.66%	1.75%	<i>flp-13(tm2448) IV.</i>	33.64%	4.33%
<i>nlp-1(ok1470) X.</i>	3.80%	1.81%	<i>ins-27(ok2474) I.</i>	39.32%	4.37%
<i>nlp-20(ok1591) IV.</i>	6.05%	0.91%	<i>nlp-17(ok3461) IV.</i>	42.33%	2.45%
<i>ins-3(ok2488) II.</i>	7.55%	1.71%	<i>ins-16(ok2919) III.</i>	43.28%	6.25%
<i>ins-11(tm1053) II.</i>	7.80%	3.77%	<i>ins-6(tm2416) II.</i>	44.31%	10.48%
<i>flp-6(ok3056) V.</i>	8.05%	6.57%	<i>ins-28(ok2722) I.</i>	46.89%	7.59%
Wild type (N2)	8.27%	1.06%	<i>ins-34(tm3095) IV.</i>	48.15%	3.21%
<i>ins-30(ok2343) I.</i>	8.76%	2.14%	<i>npr-1 (ky13) X.</i>	52.31%	4.01%
<i>flp-19(ok2460) X.</i>	8.90%	3.84%	<i>nlp-9(tm3572) V.</i>	53.66%	1.69%
<i>flp-19(ok2461) X.</i>	8.93%	2.75%	<i>ins-15(ok3444) II.</i>	59.42%	6.59%
<i>nlp-1(ok1469) X.</i>	9.13%	2.72%	<i>flp-20(ok2964) X.</i>	94.05%	2.57%
<i>flp-25(gk1016) III.</i>	9.52%	4.24%		Set 2	
<i>flp-12(ok2409) X.</i>	9.58%	0.78%	<i>egl-3 (nr2090) V.</i>	5.65%	2.12%
<i>flp-33(gk1038) I.</i>	9.71%	1.15%	Wild type (N2)	8.66%	6.16%
<i>flp-33(gk1037) I.</i>	10.09%	3.38%	<i>ins-13(tm4856) II.</i>	11.41%	2.83%
<i>ins-12(tm2918)</i>	11.05%	3.70%	<i>ins-2(tm4467) II.</i>	12.28%	3.54%
<i>flp-9(ok2730) IV.</i>	11.81%	1.35%	<i>ins-29(tm1922) I.</i>	13.86%	3.89%
<i>nlp-15(ok1512) I.</i>	12.74%	1.33%	<i>nlp-37/pdf-2(tm4393) X.</i>	18.11%	7.37%
<i>flp-10(ok2624) IV.</i>	12.81%	4.41%	<i>ins-7(tm1907) IV.</i>	21.00%	7.05%
<i>ins-18(ok1672) I.</i>	14.08%	2.46%	<i>nlp-24(tm2105) V.</i>	22.12%	3.79%
<i>flp-7(ok2625) X.</i>	14.64%	0.14%	<i>nlp-29(tm1931) V.</i>	36.29%	4.38%
<i>flp-28(gk1075)</i>	15.98%	5.68%	<i>nlp-14(tm1880) X.</i>	37.29%	7.10%
<i>ins-4(ok3534) II.</i>	17.69%	7.90%	<i>flp-27(tm4612) II.</i>	45.16%	12.21%
<i>ins-31(ok3543) II.</i>	18.66%	3.39%	<i>ins-1(tm1888) IV.</i>	49.26%	11.40%
<i>nlp-7(tm2984) X.</i>	19.37%	3.69%	<i>ins-23(tm1875) III.</i>	54.14%	11.86%
<i>ins-35(ok3297) V.</i>	21.51%	3.52%	<i>npr-1 (ky13) X.</i>	56.65%	7.75%
<i>ins-22(ok3616) III.</i>	21.62%	5.18%	<i>ins-26(tm1983) I.</i>	60.86%	7.96%
<i>ins-33 (tm2988)</i>	23.29%	8.77%	<i>nlp-2(tm1908) X.</i>	64.59%	4.04%
Larger than Wild type ($p < 0.5$) and not smaller than <i>npr-1</i> ($p > 0.05$)					
Larger than Wild type ($p < 0.5$), but smaller than <i>npr-1</i> ($p < 0.05$)					

Table B.1. Neuropeptide mutant screen: *hic* (hypersensitivity to inhibitors of cholinesterase) genes. The aldicarb-induced paralysis of worms was scored at 70 minutes. Average percentage and SEM of paralyzed worms at 70 minutes after the onset of aldicarb treatment are indicated for each mutant. 27 out of 51 genes (55 alleles) were identified whose inactivation caused significantly higher aldicarb sensitivity than wild type controls ($p < 0.05$, two tailed student t test) (indicated in green and yellow), and 16 out of the 27 mutants exhibited statistically similar extent of aldicarb hypersensitivity to *npr-1* mutants (*hic* controls) ($p > 0.05$ compared to *npr-1* mutants, student t test) (indicated in yellow).

Table B.2

% Paralyzed at 120 min					
Set 1					
Gene (allele)	Average	SEM	Gene (allele)	Average	SEM
<i>flp-20(ok2964)</i> X.	100.00%	0.00%	<i>flp-33(gk1038)</i> I.	79.78%	5.41%
<i>ins-15(ok3444)</i> II.	100.00%	0.00%	<i>ins-30(ok2343)</i> I.	79.48%	6.98%
<i>npr-1 (ky13)</i> X.	98.91%	0.72%	<i>flp-19(ok2460)</i> X.	76.57%	5.12%
<i>ins-28(ok2722)</i> I.	98.81%	1.19%	<i>ins-12(tm2918)</i>	74.47%	16.06%
<i>ins-27(ok2474)</i> I.	98.77%	1.23%	<i>ins-4(ok3534)</i> II.	72.79%	13.42%
<i>ins-34(tm3095)</i> IV.	98.67%	1.33%	<i>flp-10(ok2624)</i> IV.	72.00%	5.29%
<i>nlp-9(tm3572)</i> V.	97.53%	1.24%	<i>ins-3(ok2478)</i> II.	71.52%	13.55%
<i>ins-6(tm2416)</i> II.	97.44%	2.56%	<i>nlp-15(ok1512)</i> I.	70.32%	11.95%
<i>ins-16(ok2919)</i> III.	97.33%	2.67%	<i>flp-6(ok3056)</i> V.	66.27%	25.33%
<i>flp-7(ok2625)</i> X.	96.30%	2.14%	<i>nlp-1(ok1469)</i> X.	60.36%	7.24%
<i>nlp-7(tm2984)</i> X.	94.77%	3.55%	<i>nlp-1(ok1470)</i> X.	56.60%	13.64%
<i>ins-17(tm1790)</i> III.	94.32%	1.55%	<i>ins-3(ok2488)</i> II.	40.22%	11.43%
<i>flp-15(gk1186)</i> III.	93.83%	3.27%	<i>flp-3(ok3265)</i> X.	29.95%	8.95%
<i>ins-22(ok3616)</i> III.	93.49%	3.41%	<i>egl-3 (nr2090)</i> V.	23.65%	2.57%
<i>flp-9(ok2730)</i> IV.	92.26%	3.55%		Set 2	
<i>nlp-17(ok3461)</i> IV.	91.67%	8.33%	<i>npr-1 (ky13)</i> X.	97.71%	1.33%
<i>nlp-20(ok1591)</i> IV.	91.35%	3.32%	<i>flp-27(tm4612)</i> II.	97.19%	1.78%
<i>ins-31(ok3543)</i> II.	91.12%	5.18%	<i>nlp-2(tm1908)</i> X.	97.06%	0.99%
<i>ins-33 (tm2988)</i>	90.95%	5.85%	<i>nlp-24(tm2105)</i> V.	96.90%	1.05%
<i>ins-11(tm1053)</i> II.	90.30%	4.85%	<i>ins-26(tm1983)</i> I.	95.92%	1.70%
<i>flp-19(ok2461)</i> X.	89.83%	3.32%	<i>ins-23(tm1875)</i> III.	95.90%	2.37%
<i>flp-13(tm2448)</i> IV.	89.54%	3.55%	<i>ins-1(tm1888)</i> IV.	95.40%	0.83%
<i>flp-28(gk1075)</i>	89.28%	5.54%	<i>nlp-14(tm1880)</i> X.	94.32%	1.11%
<i>flp-16(ok3085)</i> II.	88.79%	3.66%	<i>nlp-29(tm1931)</i> V.	92.08%	3.27%
<i>nlp-18(ok1557)</i> II.	88.10%	6.30%	<i>nlp-37/pdf-2(tm4393)</i> X.	91.67%	3.16%
<i>flp-33(gk1037)</i> I.	88.03%	5.99%	<i>ins-29(tm1922)</i> I.	89.58%	1.38%
<i>flp-12(ok2409)</i> X.	86.58%	5.28%	<i>Wild type (N2)</i>	87.08%	4.62%
<i>ins-18(ok1672)</i> I.	85.83%	4.52%	<i>ins-13(tm4856)</i> II.	86.06%	4.45%
<i>ins-35(ok3297)</i> V.	85.20%	5.27%	<i>ins-7(tm1907)</i> IV.	85.64%	4.41%
<i>flp-25(gk1016)</i> III.	84.33%	10.06%	<i>ins-2(tm4467)</i> II.	77.96%	8.10%
<i>Wild type (N2)</i>	83.77%	3.15%	<i>egl-3 (nr2090)</i> V.	16.45%	1.37%
Smaller than Wild type ($p < 0.5$) and not larger than <i>npr-1</i> ($p > 0.05$)					
Smaller than Wild type ($p < 0.5$), but larger than <i>npr-1</i> ($p < 0.05$)					

Table B.2. Neuropeptide mutant screen: *ric* (resistance to inhibitors of cholinesterase) genes. The aldicarb-induced paralysis of worms was scored at 120 minutes. Average percentage and SEM of paralyzed worms at 120 minutes after the onset of aldicarb treatment are indicated for each mutant. 3 (4 alleles) out of 51 genes (55 alleles) were identified whose inactivation caused significantly lower aldicarb sensitivity than wild type controls ($p < 0.05$, two tailed student t test) (indicated in green and yellow), and 2 out of the 3 mutants exhibited statistically similar extent of aldicarb resistance to *egl-3* mutants (*ric* controls) ($p > 0.05$ compared to *egl-3* mutants, student t test) (indicated in yellow).

not outcrossed to wild type (N2 Bristol) strains after mutagenesis. Therefore, many of those mutant strains may have background mutations that can contribute to alteration in aldicarb sensitivity. To validate the primary screen results, 6 out 16 strong positive *hic* mutants and 2 out of 3 strong positive *ric* mutants were 4 times outcrossed to wild type (N2 Bristol) strains, and time course of paralysis on 1 mM aldicarb was analyzed every 10 minute for 120 minutes for each mutant. 3 mutants (*ins-6*, *ins-27*, and *nlp-9*) were confirmed to exhibit hypersensitivity to aldicarb, whereas the other 3 positive *hic* mutants and 2 positive *ric* mutants had no effect on aldicarb sensitivity (Figure B.1). Inactivating NLP-9 did not worsen the *npr-1* aldicarb hypersensitivity (Figure B.2), suggesting a possibility that NPR-1 and NLP-9 act in the same genetic pathway to inhibit cholinergic transmission at NMJs, although electrophysiological recordings should confirm this idea.

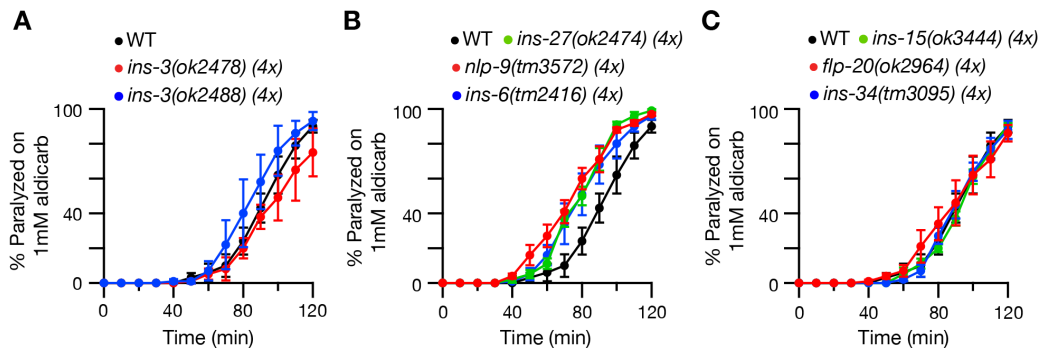


Figure B.1. Aldicarb sensitivity of outcrossed neuropeptide mutants.

(A-C) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=4 trials for wild-type, *ins-6(tm2416)*, *ins-27(ok2474)*, *nlp-9(tm3572)*, and *flp-20(ok2964)*, and n=3 trials for *ins-3(ok2478)* and *ok2488*, *ins-15(ok3444)*, and *ins-34(tm3095)*, and 20-30 worms per trial). Mutants lacking INS-6, INS-27, and NLP-9 were hypersensitive to aldicarb. Mutations inactivating FLP-20, INS-3, INS-15, and INS-34 had no effect on aldicarb sensitivity.

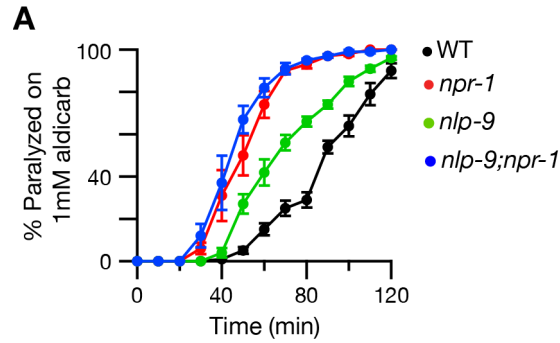


Figure B.2. NPR-1 and NLP-9 inhibit aldicarb sensitivity in the same genetic pathway. (A) Time courses of paralysis of worms on 1 mM aldicarb were plotted for the indicated genotypes (n=8 trials for all the genotypes, and 20-30 worms per trial; wild-type, *npr-1(ky13)*, *nlp-9(tm3572)*, and *nlp-9;npr-1*). *npr-1* single mutants and *nlp-9;npr-1* double mutants showed a similar extent of aldicarb hypersensitivity.

REFERENCES

- Allada, R., and Chung, B.Y. (2010). Circadian organization of behavior and physiology in *Drosophila*. *Annu Rev Physiol* 72, 605-624.
- Antonijevic, I.A., Murck, H., Bohlhalter, S., Frieboes, R.M., Holsboer, F., and Steiger, A. (2000). Neuropeptide Y promotes sleep and inhibits ACTH and cortisol release in young men. *Neuropharmacology* 39, 1474-1481.
- Aronoff, R., Mellem, J.E., Maricq, A.V., Sprengel, R., and Seeburg, P.H. (2004). Neuronal toxicity in *Caenorhabditis elegans* from an editing site mutant in glutamate receptor channels. *J Neurosci* 24, 8135-8140.
- Babu, K., Hu, Z., Chien, S.C., Garriga, G., and Kaplan, J.M. (2011). The immunoglobulin super family protein RIG-3 prevents synaptic potentiation and regulates Wnt signaling. *Neuron* 71, 103-116.
- Bai, J., Hu, Z., Dittman, J.S., Pym, E.C., and Kaplan, J.M. (2010). Endophilin functions as a membrane-bending molecule and is delivered to endocytic zones by exocytosis. *Cell* 143, 430-441.
- Bale, T.L., and Vale, W.W. (2004). CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu Rev Pharmacol Toxicol* 44, 525-557.
- Bargmann, C.I., Hartweg, E., and Horvitz, H.R. (1993). Odorant-selective genes and neurons mediate olfaction in *C. elegans*. *Cell* 74, 515-527.
- Barrios, A., Ghosh, R., Fang, C., Emmons, S.W., and Barr, M.M. (2012). PDF-1 neuropeptide signaling modulates a neural circuit for mate-searching behavior in *C. elegans*. *Nat Neurosci* 15, 1675-1682.
- Beets, I., Janssen, T., Meelkop, E., Temmerman, L., Suetens, N., Rademakers, S., Jansen, G., and Schoofs, L. (2012). Vasopressin/oxytocin-related signaling regulates gustatory associative learning in *C. elegans*. *Science* 338, 543-545.
- Borbely, A.A. (1982). A two process model of sleep regulation. *Hum Neurobiol* 1, 195-204.

- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71-94.
- Bretscher, A.J., Busch, K.E., and de Bono, M. (2008). A carbon dioxide avoidance behavior is integrated with responses to ambient oxygen and food in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* 105, 8044-8049.
- Brockie, P.J., Madsen, D.M., Zheng, Y., Mellem, J., and Maricq, A.V. (2001). Differential expression of glutamate receptor subunits in the nervous system of *Caenorhabditis elegans* and their regulation by the homeodomain protein UNC-42. *J Neurosci* 21, 1510-1522.
- Brockie, P.J., and Maricq, A.V. (2006). Ionotropic glutamate receptors: genetics, behavior and electrophysiology. *WormBook*, 1-16.
- Bushey, D., Tononi, G., and Cirelli, C. (2011). Sleep and synaptic homeostasis: structural evidence in *Drosophila*. *Science* 332, 1576-1581.
- Cassada, R.C., and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Dev Biol* 46, 326-342.
- Chalasani, S.H., Kato, S., Albrecht, D.R., Nakagawa, T., Abbott, L.F., and Bargmann, C.I. (2010). Neuropeptide feedback modifies odor-evoked dynamics in *Caenorhabditis elegans* olfactory neurons. *Nat Neurosci* 13, 615-621.
- Chalfie, M., and Sulston, J. (1981). Developmental genetics of the mechanosensory neurons of *Caenorhabditis elegans*. *Dev Biol* 82, 358-370.
- Chalfie, M., Sulston, J.E., White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1985). The neural circuit for touch sensitivity in *Caenorhabditis elegans*. *J Neurosci* 5, 956-964.
- Chemelli, R.M., Willie, J.T., Sinton, C.M., Elmquist, J.K., Scammell, T., Lee, C., Richardson, J.A., Williams, S.C., Xiong, Y., Kisanuki, Y., *et al.* (1999). Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 98, 437-451.
- Cheung, B.H., Arellano-Carbajal, F., Rybicki, I., and de Bono, M. (2004). Soluble guanylate cyclases act in neurons exposed to the body fluid to promote *C. elegans* aggregation behavior. *Curr Biol* 14, 1105-1111.

- Cheung, B.H., Cohen, M., Rogers, C., Albayram, O., and de Bono, M. (2005). Experience-dependent modulation of *C. elegans* behavior by ambient oxygen. *Curr Biol* *15*, 905-917.
- Cirelli, C. (2009). The genetic and molecular regulation of sleep: from fruit flies to humans. *Nat Rev Neurosci* *10*, 549-560.
- Coates, J.C., and de Bono, M. (2002). Antagonistic pathways in neurons exposed to body fluid regulate social feeding in *Caenorhabditis elegans*. *Nature* *419*, 925-929.
- de Bono, M., and Bargmann, C.I. (1998). Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in *C. elegans*. *Cell* *94*, 679-689.
- de Bono, M., Tobin, D.M., Davis, M.W., Avery, L., and Bargmann, C.I. (2002). Social feeding in *Caenorhabditis elegans* is induced by neurons that detect aversive stimuli. *Nature* *419*, 899-903.
- Dyzma, M., Boudjeltia, K.Z., Faraut, B., and Kerkhofs, M. (2010). Neuropeptide Y and sleep. *Sleep Med Rev* *14*, 161-165.
- Ehlers, C.L., Somes, C., Seifritz, E., and Rivier, J.E. (1997). CRF/NPY interactions: a potential role in sleep dysregulation in depression and anxiety. *Depress Anxiety* *6*, 1-9.
- Fares, H., and Greenwald, I. (2001). Genetic analysis of endocytosis in *Caenorhabditis elegans*: coelomocyte uptake defective mutants. *Genetics* *159*, 133-145.
- Feng, G., Reale, V., Chatwin, H., Kennedy, K., Venard, R., Ericsson, C., Yu, K., Evans, P.D., and Hall, L.M. (2003). Functional characterization of a neuropeptide F-like receptor from *Drosophila melanogaster*. *Eur J Neurosci* *18*, 227-238.
- Frand, A.R., Russel, S., and Ruvkun, G. (2005). Functional genomic analysis of *C. elegans* molting. *PLoS Biol* *3*, e312.
- Fu, L.Y., Acuna-Goycolea, C., and van den Pol, A.N. (2004). Neuropeptide Y inhibits hypocretin/orexin neurons by multiple presynaptic and postsynaptic mechanisms: tonic depression of the hypothalamic arousal system. *J Neurosci* *24*, 8741-8751.

- Garrison, J.L., Macosko, E.Z., Bernstein, S., Pokala, N., Albrecht, D.R., and Bargmann, C.I. (2012). Oxytocin/vasopressin-related peptides have an ancient role in reproductive behavior. *Science* 338, 540-543.
- Gilestro, G.F., Tononi, G., and Cirelli, C. (2009). Widespread changes in synaptic markers as a function of sleep and wakefulness in *Drosophila*. *Science* 324, 109-112.
- Gracheva, E.O., Burdina, A.O., Holgado, A.M., Berthelot-Grosjean, M., Ackley, B.D., Hadwiger, G., Nonet, M.L., Weimer, R.M., and Richmond, J.E. (2006). Tomosyn inhibits synaptic vesicle priming in *Caenorhabditis elegans*. *PLoS Biol* 4, e261.
- Gray, J.M., Karow, D.S., Lu, H., Chang, A.J., Chang, J.S., Ellis, R.E., Marletta, M.A., and Bargmann, C.I. (2004). Oxygen sensation and social feeding mediated by a *C. elegans* guanylate cyclase homologue. *Nature* 430, 317-322.
- Hallam, E.A., and Sternberg, P.W. (2008). Acute carbon dioxide avoidance in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* 105, 8038-8043.
- Hart, A.C., Kass, J., Shapiro, J.E., and Kaplan, J.M. (1999). Distinct signaling pathways mediate touch and osmosensory responses in a polymodal sensory neuron. *J Neurosci* 19, 1952-1958.
- Hart, A.C., Sims, S., and Kaplan, J.M. (1995). Synaptic code for sensory modalities revealed by *C. elegans* GLR-1 glutamate receptor. *Nature* 378, 82-85.
- Heilig, M., and Murison, R. (1987). Intracerebroventricular neuropeptide Y suppresses open field and home cage activity in the rat. *Regul Pept* 19, 221-231.
- Held, K., Antonijevic, I., Murck, H., Kuenzel, H., and Steiger, A. (2006). Neuropeptide Y (NPY) shortens sleep latency but does not suppress ACTH and cortisol in depressed patients and normal controls. *Psychoneuroendocrinology* 31, 100-107.
- Helfrich-Forster, C. (1995). The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* 92, 612-616.
- Hensch, T.K. (2005). Critical period plasticity in local cortical circuits. *Nat Rev Neurosci* 6, 877-888.

Hilliard, M.A., Bergamasco, C., Arbucci, S., Plasterk, R.H., and Bazzicalupo, P. (2004). Worms taste bitter: ASH neurons, QUI-1, GPA-3 and ODR-3 mediate quinine avoidance in *Caenorhabditis elegans*. *Embo J* 23, 1101-1111.

Hinuma, S., Shintani, Y., Fukusumi, S., Iijima, N., Matsumoto, Y., Hosoya, M., Fujii, R., Watanabe, T., Kikuchi, K., Terao, Y., *et al.* (2000). New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. *Nature cell biology* 2, 703-708.

Holtmaat, A., and Svoboda, K. (2009). Experience-dependent structural synaptic plasticity in the mammalian brain. *Nat Rev Neurosci* 10, 647-658.

Hu, W.P., Li, J.D., Colwell, C.S., and Zhou, Q.Y. (2011a). Decreased REM sleep and altered circadian sleep regulation in mice lacking vasoactive intestinal polypeptide. *Sleep* 34, 49-56.

Hu, Z., Pym, E.C., Babu, K., Vashlishan Murray, A.B., and Kaplan, J.M. (2011b). A neuropeptide-mediated stretch response links muscle contraction to changes in neurotransmitter release. *Neuron* 71, 92-102.

Husson, S.J., Clynen, E., Baggerman, G., Janssen, T., and Schoofs, L. (2006). Defective processing of neuropeptide precursors in *Caenorhabditis elegans* lacking proprotein convertase 2 (KPC-2/EGL-3): mutant analysis by mass spectrometry. *J Neurochem* 98, 1999-2012.

Husson, S.J., Janssen, T., Baggerman, G., Bogert, B., Kahn-Kirby, A.H., Ashrafi, K., and Schoofs, L. (2007). Impaired processing of FLP and NLP peptides in carboxypeptidase E (EGL-21)-deficient *Caenorhabditis elegans* as analyzed by mass spectrometry. *J Neurochem* 102, 246-260.

Husson, S.J., and Schoofs, L. (2007). Altered neuropeptide profile of *Caenorhabditis elegans* lacking the chaperone protein 7B2 as analyzed by mass spectrometry. *FEBS Lett* 581, 4288-4292.

Imeri, L., and Opp, M.R. (2009). How (and why) the immune system makes us sleep. *Nat Rev Neurosci* 10, 199-210.

Jacob, T.C., and Kaplan, J.M. (2003). The EGL-21 carboxypeptidase E facilitates acetylcholine release at *Caenorhabditis elegans* neuromuscular junctions. *J Neurosci* 23, 2122-2130.

- Jang, H., Kim, K., Neal, S.J., Macosko, E., Kim, D., Butcher, R.A., Zeiger, D.M., Bargmann, C.I., and Sengupta, P. (2012). Neuromodulatory state and sex specify alternative behaviors through antagonistic synaptic pathways in *C. elegans*. *Neuron* 75, 585-592.
- Janssen, T., Husson, S.J., Lindemans, M., Mertens, I., Rademakers, S., Ver Donck, K., Geysen, J., Jansen, G., and Schoofs, L. (2008). Functional characterization of three G protein-coupled receptors for pigment dispersing factors in *Caenorhabditis elegans*. *J Biol Chem* 283, 15241-15249.
- Janssen, T., Husson, S.J., Meelkop, E., Temmerman, L., Lindemans, M., Verstraelen, K., Rademakers, S., Mertens, I., Nitabach, M., Jansen, G., *et al.* (2009). Discovery and characterization of a conserved pigment dispersing factor-like neuropeptide pathway in *Caenorhabditis elegans*. *J Neurochem* 111, 228-241.
- Jeon, M., Gardner, H.F., Miller, E.A., Deshler, J., and Rougvie, A.E. (1999). Similarity of the *C. elegans* developmental timing protein LIN-42 to circadian rhythm proteins. *Science* 286, 1141-1146.
- Jolicoeur, F.B., Michaud, J.N., Rivest, R., Menard, D., Gaudin, D., Fournier, A., and St-Pierre, S. (1991). Neurobehavioral profile of neuropeptide Y. *Brain Res Bull* 26, 265-268.
- Jones, B.E. (2008). Modulation of cortical activation and behavioral arousal by cholinergic and orexinergic systems. *Ann N Y Acad Sci* 1129, 26-34.
- Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., *et al.* (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421, 231-237.
- Kamp, T.J., and Hell, J.W. (2000). Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. *Circ Res* 87, 1095-1102.
- Kaplan, J.M., and Horvitz, H.R. (1993). A dual mechanosensory and chemosensory neuron in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* 90, 2227-2231.
- Kass, J., Jacob, T.C., Kim, P., and Kaplan, J.M. (2001). The EGL-3 proprotein convertase regulates mechanosensory responses of *Caenorhabditis elegans*. *J Neurosci* 21, 9265-9272.

- Kim, K., Sato, K., Shibuya, M., Zeiger, D.M., Butcher, R.A., Ragains, J.R., Clardy, J., Touhara, K., and Sengupta, P. (2009). Two chemoreceptors mediate developmental effects of dauer pheromone in *C. elegans*. *Science* 326, 994-998.
- Komatsu, H., Mori, I., Rhee, J.S., Akaike, N., and Ohshima, Y. (1996). Mutations in a cyclic nucleotide-gated channel lead to abnormal thermosensation and chemosensation in *C. elegans*. *Neuron* 17, 707-718.
- Krueger, J.M., and Majde, J.A. (1994). Microbial products and cytokines in sleep and fever regulation. *Crit Rev Immunol* 14, 355-379.
- Krueger, J.M., Obal, F.J., Fang, J., Kubota, T., and Taishi, P. (2001). The role of cytokines in physiological sleep regulation. *Ann N Y Acad Sci* 933, 211-221.
- Kubiak, T.M., Larsen, M.J., Nulf, S.C., Zantello, M.R., Burton, K.J., Bowman, J.W., Modric, T., and Lowery, D.E. (2003). Differential activation of "social" and "solitary" variants of the *Caenorhabditis elegans* G protein-coupled receptor NPR-1 by its cognate ligand AF9. *J Biol Chem* 278, 33724-33729.
- Lee, R.Y., Sawin, E.R., Chalfie, M., Horvitz, H.R., and Avery, L. (1999). EAT-4, a homolog of a mammalian sodium-dependent inorganic phosphate cotransporter, is necessary for glutamatergic neurotransmission in *caenorhabditis elegans*. *J Neurosci* 19, 159-167.
- Li, C., and Kim, K. (2008). Neuropeptides. *WormBook*, 1-36.
- Lin, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., Qiu, X., de Jong, P.J., Nishino, S., and Mignot, E. (1999). The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 98, 365-376.
- Lindberg, I., Tu, B., Muller, L., and Dickerson, I.M. (1998). Cloning and functional analysis of *C. elegans* 7B2. *DNA Cell Biol* 17, 727-734.
- Loria, P.M., Hodgkin, J., and Hobert, O. (2004). A conserved postsynaptic transmembrane protein affecting neuromuscular signaling in *Caenorhabditis elegans*. *J Neurosci* 24, 2191-2201.

Macosko, E.Z., Pokala, N., Feinberg, E.H., Chalasani, S.H., Butcher, R.A., Clardy, J., and Bargmann, C.I. (2009). A hub-and-spoke circuit drives pheromone attraction and social behaviour in *C. elegans*. *Nature* 458, 1171-1175.

Marder, E. (2012). Neuromodulation of neuronal circuits: back to the future. *Neuron* 76, 1-11.

Maricq, A.V., Peckol, E., Driscoll, M., and Bargmann, C.I. (1995). Mechanosensory signalling in *C. elegans* mediated by the GLR-1 glutamate receptor. *Nature* 378, 78-81.

Maywood, E.S., O'Neill, J.S., Chesham, J.E., and Hastings, M.H. (2007). Minireview: The circadian clockwork of the suprachiasmatic nuclei--analysis of a cellular oscillator that drives endocrine rhythms. *Endocrinology* 148, 5624-5634.

McEwen, J.M., Madison, J.M., Dybbs, M., and Kaplan, J.M. (2006). Antagonistic regulation of synaptic vesicle priming by Tomosyn and UNC-13. *Neuron* 51, 303-315.

McGrath, P.T., Rockman, M.V., Zimmer, M., Jang, H., Macosko, E.Z., Kruglyak, L., and Bargmann, C.I. (2009). Quantitative mapping of a digenic behavioral trait implicates globin variation in *C. elegans* sensory behaviors. *Neuron* 61, 692-699.

McGrath, P.T., Xu, Y., Ailion, M., Garrison, J.L., Butcher, R.A., and Bargmann, C.I. (2011). Parallel evolution of domesticated *Caenorhabditis* species targets pheromone receptor genes. *Nature* 477, 321-325.

Meelkop, E., Temmerman, L., Janssen, T., Suetens, N., Beets, I., Van Rompay, L., Shanmugam, N., Husson, S.J., and Schoofs, L. (2012). PDF receptor signaling in *Caenorhabditis elegans* modulates locomotion and egg-laying. *Mol Cell Endocrinol* 361, 232-240.

Mellem, J.E., Brockie, P.J., Zheng, Y., Madsen, D.M., and Maricq, A.V. (2002). Decoding of polymodal sensory stimuli by postsynaptic glutamate receptors in *C. elegans*. *Neuron* 36, 933-944.

Miller, K.G., Emerson, M.D., and Rand, J.B. (1999). Gqalpha and diacylglycerol kinase negatively regulate the Gqalpha pathway in *C. elegans*. *Neuron* 24, 323-333.

Monsalve, G.C., Van Buskirk, C., and Frand, A.R. (2011). LIN-42/PERIOD controls cyclical and developmental progression of *C. elegans* molts. *Curr Biol* 21, 2033-2045.

Moss, E.G. (2007). Heterochronic genes and the nature of developmental time. *Curr Biol* 17, R425-434.

Nishino, S., Ripley, B., Overeem, S., Lammers, G.J., and Mignot, E. (2000). Hypocretin (orexin) deficiency in human narcolepsy. *Lancet* 355, 39-40.

Nitz, D.A., van Swinderen, B., Tononi, G., and Greenspan, R.J. (2002). Electrophysiological correlates of rest and activity in *Drosophila melanogaster*. *Curr Biol* 12, 1934-1940.

Nurrish, S., Segalat, L., and Kaplan, J.M. (1999). Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites. *Neuron* 24, 231-242.

Palmiter, R.D. (2008). Dopamine signaling in the dorsal striatum is essential for motivated behaviors: lessons from dopamine-deficient mice. *Ann N Y Acad Sci* 1129, 35-46.

Parisky, K.M., Agosto, J., Pulver, S.R., Shang, Y., Kuklin, E., Hodge, J.J., Kang, K., Liu, X., Garrity, P.A., Rosbash, M., *et al.* (2008). PDF cells are a GABA-responsive wake-promoting component of the *Drosophila* sleep circuit. *Neuron* 60, 672-682.

Park, J.H., and Hall, J.C. (1998). Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster*. *J Biol Rhythms* 13, 219-228.

Peyron, C., Faraco, J., Rogers, W., Ripley, B., Overeem, S., Charnay, Y., Nevsimalova, S., Aldrich, M., Reynolds, D., Albin, R., *et al.* (2000). A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 6, 991-997.

Pfaff, D., Ribeiro, A., Matthews, J., and Kow, L.M. (2008a). Concepts and mechanisms of generalized central nervous system arousal. *Ann N Y Acad Sci* 1129, 11-25.

Pfaff, D.W. (2006). Brain arousal and information theory : neural and genetic mechanisms (Cambridge, Mass., Harvard University Press).

Pfaff, D.W., Kieffer, B.L., and Swanson, L.W. (2008b). Mechanisms for the regulation of state changes in the central nervous system: an introduction. *Ann N Y Acad Sci* 1129, 1-7.

Prober, D.A., Rihel, J., Onah, A.A., Sung, R.J., and Schier, A.F. (2006). Hypocretin/orexin overexpression induces an insomnia-like phenotype in zebrafish. *J Neurosci* 26, 13400-13410.

Raizen, D.M., Zimmerman, J.E., Maycock, M.H., Ta, U.D., You, Y.J., Sundaram, M.V., and Pack, A.I. (2008). Lethargus is a *Caenorhabditis elegans* sleep-like state. *Nature* 451, 569-572.

Reddy, K.C., Andersen, E.C., Kruglyak, L., and Kim, D.H. (2009). A polymorphism in *npr-1* is a behavioral determinant of pathogen susceptibility in *C. elegans*. *Science* 323, 382-384.

Renn, S.C., Park, J.H., Rosbash, M., Hall, J.C., and Taghert, P.H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* 99, 791-802.

Richmond, J.E., Davis, W.S., and Jorgensen, E.M. (1999). UNC-13 is required for synaptic vesicle fusion in *C. elegans*. *Nat Neurosci* 2, 959-964.

Rogers, C., Reale, V., Kim, K., Chatwin, H., Li, C., Evans, P., and de Bono, M. (2003). Inhibition of *Caenorhabditis elegans* social feeding by FMRFamide-related peptide activation of NPR-1. *Nat Neurosci* 6, 1178-1185.

Sambongi, Y., Nagae, T., Liu, Y., Yoshimizu, T., Takeda, K., Wada, Y., and Futai, M. (1999). Sensing of cadmium and copper ions by externally exposed ADL, ASE, and ASH neurons elicits avoidance response in *Caenorhabditis elegans*. *Neuroreport* 10, 753-757.

Schmitz, C., Kinge, P., and Hutter, H. (2007). Axon guidance genes identified in a large-scale RNAi screen using the RNAi-hypersensitive *Caenorhabditis elegans* strain *nre-1(hd20) lin-15b(hd126)*. *Proc Natl Acad Sci U S A* 104, 834-839.

Schwarz, J., Lewandrowski, I., and Bringmann, H. (2011). Reduced activity of a sensory neuron during a sleep-like state in *Caenorhabditis elegans*. *Curr Biol* 21, R983-984.

- Segalat, L., Elkes, D.A., and Kaplan, J.M. (1995). Modulation of serotonin-controlled behaviors by Go in *Caenorhabditis elegans*. *Science* 267, 1648-1651.
- Sieburth, D., Ch'ng, Q., Dybbs, M., Tavazoie, M., Kennedy, S., Wang, D., Dupuy, D., Rual, J.F., Hill, D.E., Vidal, M., *et al.* (2005). Systematic analysis of genes required for synapse structure and function. *Nature* 436, 510-517.
- Sieburth, D., Madison, J.M., and Kaplan, J.M. (2007). PKC-1 regulates secretion of neuropeptides. *Nat Neurosci* 10, 49-57.
- Silver, R., and Lesauter, J. (2008). Circadian and homeostatic factors in arousal. *Ann N Y Acad Sci* 1129, 263-274.
- Singh, K., Chao, M.Y., Somers, G.A., Komatsu, H., Corkins, M.E., Larkins-Ford, J., Tucey, T., Dionne, H.M., Walsh, M.B., Beaumont, E.K., *et al.* (2011). *C. elegans* Notch signaling regulates adult chemosensory response and larval molting quiescence. *Curr Biol* 21, 825-834.
- Smith, G.W., Aubry, J.M., Dellu, F., Contarino, A., Bilezikjian, L.M., Gold, L.H., Chen, R., Marchuk, Y., Hauser, C., Bentley, C.A., *et al.* (1998). Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron* 20, 1093-1102.
- Srinivasan, J., von Reuss, S.H., Bose, N., Zaslaver, A., Mahanti, P., Ho, M.C., O'Doherty, O.G., Edison, A.S., Sternberg, P.W., and Schroeder, F.C. (2012). A modular library of small molecule signals regulates social behaviors in *Caenorhabditis elegans*. *PLoS Biol* 10, e1001237.
- Stenzel-Poore, M.P., Heinrichs, S.C., Rivest, S., Koob, G.F., and Vale, W.W. (1994). Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. *J Neurosci* 14, 2579-2584.
- Steriade, M., McCormick, D.A., and Sejnowski, T.J. (1993). Thalamocortical oscillations in the sleeping and aroused brain. *Science* 262, 679-685.
- Styer, K.L., Singh, V., Macosko, E., Steele, S.E., Bargmann, C.I., and Aballay, A. (2008). Innate immunity in *Caenorhabditis elegans* is regulated by neurons expressing NPR-1/GPCR. *Science* 322, 460-464.

Sun, L., and Miller, R.J. (1999). Multiple neuropeptide Y receptors regulate K⁺ and Ca²⁺ channels in acutely isolated neurons from the rat arcuate nucleus. *J Neurophysiol* 81, 1391-1403.

Sun, Q.Q., Baraban, S.C., Prince, D.A., and Huguenard, J.R. (2003). Target-specific neuropeptide Y-ergic synaptic inhibition and its network consequences within the mammalian thalamus. *J Neurosci* 23, 9639-9649.

Sun, Q.Q., Huguenard, J.R., and Prince, D.A. (2001). Neuropeptide Y receptors differentially modulate G-protein-activated inwardly rectifying K⁺ channels and high-voltage-activated Ca²⁺ channels in rat thalamic neurons. *The Journal of physiology* 531, 67-79.

Sutcliffe, J.G., and de Lecea, L. (2002). The hypocretins: setting the arousal threshold. *Nat Rev Neurosci* 3, 339-349.

Suzuki, H., Kerr, R., Bianchi, L., Frokjaer-Jensen, C., Slone, D., Xue, J., Gerstbrein, B., Driscoll, M., and Schafer, W.R. (2003). In vivo imaging of *C. elegans* mechanosensory neurons demonstrates a specific role for the MEC-4 channel in the process of gentle touch sensation. *Neuron* 39, 1005-1017.

Taghert, P.H., and Nitabach, M.N. (2012). Peptide neuromodulation in invertebrate model systems. *Neuron* 76, 82-97.

Talsma, A.D., Christov, C.P., Terriente-Felix, A., Linneweber, G.A., Perea, D., Wayland, M., Shafer, O.T., and Miguel-Aliaga, I. (2012). Remote control of renal physiology by the intestinal neuropeptide pigment-dispersing factor in *Drosophila*. *Proc Natl Acad Sci U S A* 109, 12177-12182.

Timpl, P., Spanagel, R., Sillaber, I., Kresse, A., Reul, J.M., Stalla, G.K., Blanquet, V., Steckler, T., Holsboer, F., and Wurst, W. (1998). Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nature genetics* 19, 162-166.

Tobin, D., Madsen, D., Kahn-Kirby, A., Peckol, E., Moulder, G., Barstead, R., Maricq, A., and Bargmann, C. (2002). Combinatorial expression of TRPV channel proteins defines their sensory functions and subcellular localization in *C. elegans* neurons. *Neuron* 35, 307-318.

- Troemel, E.R., Chou, J.H., Dwyer, N.D., Colbert, H.A., and Bargmann, C.I. (1995). Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. *Cell* 83, 207-218.
- Troemel, E.R., Kimmel, B.E., and Bargmann, C.I. (1997). Reprogramming chemotaxis responses: sensory neurons define olfactory preferences in *C. elegans*. *Cell* 91, 161-169.
- Van Buskirk, C., and Sternberg, P.W. (2007). Epidermal growth factor signaling induces behavioral quiescence in *Caenorhabditis elegans*. *Nat Neurosci* 10, 1300-1307.
- van den Pol, A.N. (2012). Neuropeptide transmission in brain circuits. *Neuron* 76, 98-115.
- Vashlishan, A.B., Madison, J.M., Dybbs, M., Bai, J., Sieburth, D., Ch'ng, Q., Tavazoie, M., and Kaplan, J.M. (2008). An RNAi screen identifies genes that regulate GABA synapses. *Neuron* 58, 346-361.
- Vyazovskiy, V.V., Cirelli, C., Pfister-Genskow, M., Faraguna, U., and Tononi, G. (2008). Molecular and electrophysiological evidence for net synaptic potentiation in wake and depression in sleep. *Nat Neurosci* 11, 200-208.
- Way, J.C., and Chalfie, M. (1988). *mec-3*, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in *C. elegans*. *Cell* 54, 5-16.
- Weber, K.P., De, S., Kozarewa, I., Turner, D.J., Babu, M.M., and de Bono, M. (2010). Whole genome sequencing highlights genetic changes associated with laboratory domestication of *C. elegans*. *PLoS One* 5, e13922.
- White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 314, 1-340.
- Wiesel, T.N., and Hubel, D.H. (1963). Single-Cell Responses in Striate Cortex of Kittens Deprived of Vision in One Eye. *J Neurophysiol* 26, 1003-1017.
- Wu, Q., Wen, T., Lee, G., Park, J.H., Cai, H.N., and Shen, P. (2003). Developmental control of foraging and social behavior by the *Drosophila* neuropeptide Y-like system. *Neuron* 39, 147-161.

You, Y.J., Kim, J., Raizen, D.M., and Avery, L. (2008). Insulin, cGMP, and TGF-beta signals regulate food intake and quiescence in *C. elegans*: a model for satiety. *Cell Metab* 7, 249-257.